

4 November 1982

Putting a case for civil science

The British government's Advisory Board for the Research Councils has made public its advice on spending money. In the process, it has unwittingly shown how its own work could be improved.

Even British governments, notoriously shy of giving reasons for their decisions, occasionally discover that openness does not jeopardize their survival or even offend against the laughable doctrine that only ministers of the crown take decisions. That is the most obvious lesson to be drawn from the publication last week (see page 3) of this year's advice of the Advisory Board for the Research Councils to the Secretary of the State for Education and Science, Sir Keith Joseph. Hitherto, this information has been as closely (perhaps more closely) guarded than that collected by the British intelligence services, on the grounds that it would be a great embarrassment if the responsible minister decided not to follow advice given. As it happens, Sir Keith Joseph seems to be the first minister to have disagreed with the board on how the budget for civil science should be divided. Now, to his credit, he has agreed that the advice from which he in part dissents should be open to public scrutiny. One result is a better appreciation of how these important issues are decided: too casually. Another is that the minister's hand will be strengthened in the battle to come with the British Treasury about the scale of research spending in the next three years.

The advisory board itself will benefit from the need to give persuasive arguments for its advice. Among the British government's battalion of advisory committees, the board is conspicuous because its most influential members have a direct interest in whatever advice is given. Since the board was first set up, after the Rothschild inquiry in 1971, its chief function has been to divide what the government is prepared to spend on civil science among the interested parties, principally the five research councils, each of whose chief executives is a member of the board.

The fear that if dog eats dog it may itself be bitten, frequently mistaken for gentlemanly politeness, explains why over the years the distribution of the science budget has changed only in minor ways. The Science and Engineering Research Council lost a little ground in the late 1970s, when high-energy accelerators and space research became unfashionable, but that balance is now in part to be redressed. The Medical Research Council was smart enough two years ago to recapture from the Department of Health the slice of its budget transferred to the government department in 1971. Otherwise, the advisory board has operated at the margins of its dependants' budgets, trimming a little here, adding a little there. Complacency in the face of adversity seems to have been the motto.

Publicity

Even the prospect of publicity seems to have helped to jerk the board out of this self-defeating groove. Its proposal that the British Museum (Natural History) should not now build a huge new exhibition space within the shell of its splendid Victorian building is not merely an economy (£23 million plus) but a public service. The recommendation that the budget of the Agricultural Research Council should level off in cash terms (and therefore shrink in real terms) after the next financial year will cause more serious damage. This council is more trapped than others by its commitments to its own staffs working in its own research institutes. Yet the board's case for letting agriculture carry the main burden of the economies necessary to finance future spending by the Science and Engineering Research Council would be more convincing if its own statement of priorities had been

more persuasive. That the board should attach importance to "developments in biology" is natural enough, even if superficially inconsistent with its recommendation about agricultural research. To count as a priority "new developments in neuroscience" is sensible enough; but this goal is surprisingly not mentioned in the statements with which the research councils bid for funds. Did one of the board's members meet a neurobiologist on a train journey, perhaps?

Fashion

The board's first public account of its way of working also raises the suspicion that it is as liable as the next person to be carried uncritically away by fashion. Thus the board blesses with £22.5 million (over three years) the Science and Engineering Research Council's wish to become the basic research sponsor in a "national programme on information technology" about to be launched by the Department of Industry, and which may cost the council £50 million over the next five years. The snag, for readers of the board's account of itself, is that there is no way of telling whether this expensive exercise is supported by an assessment of ways in which money might usefully be spent on research or merely by the enthusiasm which Mr Kenneth Baker, a minister of state at the Department of Industry, has brought to his job as minister of information technology. Fashion of a different kind also accounts for the choice of "remote sensing" as another priority area of research: the chairman of the Natural Environment Research Council, Sir Hermann Bondi, is keen; meteorological observations from satellites are practically important, while oceanic observations will soon be an important source of data in oceanography; and civil servants can readily be persuaded that the objective is to find oil, or tin, or some other mineral.

Fashion seems also to have infected the board's report in its uncritical endorsement of (and collaboration with) the study of the relations between industry, universities and the research councils commissioned by the British government from the Advisory Committee on Applied Research and Development. That committee's terms of reference are hopelessly uncritical — essentially, to identify obstacles to making university research a handmaiden of British industry and to suggest how they might be removed. The committee appointed for this task appears to be aware that there are dangers as well as benefits in links between academic and industrial organizations, principally that universities may be made less able to dispense impartially their special kind of knowledge to all potential users of it. The Advisory Board for the Research Councils, custodian of basic research though it professes to be, says nothing on this point. But if the board is silent on the risks in too particular a relationship between universities and industry, who else can be expected to have influence?

These are the respects in which the board's first public report is deficient. The document also has merit. Last December, the Select Committee on Science and Technology of the House of Lords (see *Nature* 294, 503; 1981) complained that the board had become too passive an instrument of policy, and the British government replied that the board should exert itself in throwing light on matters of public policy (see *Nature* 8 July, p.107). No doubt the flurry of activity there has been in the past few weeks in



setting up inquiries into topics such as the relations between research councils and universities is one sign that the board has woken up. Another is an important discussion, now published, of the demographic problems presented by the maldistribution of university teachers in most disciplines, a consequence of the rapid expansion of the 1960s. As elsewhere, there are too few young people and too many who are middle-aged (see chart, p. 8). Much of the redirections of science spending planned for the next few years is intended to redress this balance, and not before time. The obvious danger is that by using money intended for research to improve the condition of British universities, the research councils may be helping to break the board's rule that they should not attempt to make good the deficiencies of the dual-support system. The snag is that self-restraint in that regard requires not merely that the councils should back good people, but that they should do so only if those people are well placed to carry out research effectively. So why should not the board spell out the criteria it expects that its dependants will follow?

A more awkward question needs to be taken up (in next year's review?): are the board's dependants as efficient as they should be? In the past few years, the peer-review systems operated by most of the research councils have won widespread respect, but many of their own research establishments fall far short of what they might be. Perhaps the most conspicuous example is the Medical Research Council's National Institute for Medical Research, which has long been balkanized into "divisions" that hardly ever speak to each other and where even minor items of expenditure must be met by cheques paid from headquarters. The board now repeats the research council's promise that its institute will be "restructured", and has appointed Dr D. A. Rees to that unenviable task. But who is to say that this one institute is not just the visible tip of a much larger problem?

Old-fashioned ways of delegating financial responsibility are certainly not confined to one institute or even to one research council. And all those that depend to some extent on contracts with government departments for the conduct of applied research are necessarily caught up in a complicated set of negotiations that only occasionally leads to a change in the pattern of expenditure. Similarly, the recruitment of extra staff within the research council system is hampered by rules dictated from on high which are designed to limit the total strength of the public service, but which prevent research council establishments from earning honest money when they can. The British Prime Minister's favourite cost-cutter, Sir Derek Rayner, is thought recently to have turned his attention to the research councils. It will be best for everybody if he discovers that the present system is too much hamstrung from outside.

The most serious defect in the working of the advisory board, now revealed, is however the incompleteness of such authority as it enjoys even in the field with which it is concerned. (The case that there should be some mechanism for overseeing the whole of publicly-supported research, defence research included, is irresistible but nevertheless repeatedly denied.) The board's membership includes most of the chief scientists of government departments which are in their own right substantial sponsors of research, much of it in universities, little of it different in kind from work sponsored by the research councils. The British government has firmly rejected the argument of the House of Lords Select Committee that there should be some device for overseeing the whole of public sponsorship of research and development, partly on the grounds that the present prime minister has a personal interest in the subject. But can it make sense that government departments, from health to industry, should be caught up in a semi-judicial (but over-casual) assessment of what the research councils should be allowed to do when their own spending in similar fields is innocent of outside inquiry? The simple answer, which all bureaucrats will echo, is that what the advisory board calls the science budget is a charge on the Department of Education and Science, and that there would be departmental barriers to a more wide-ranging inquiry. But it makes no sense that such procedural matters should inhibit what good sense requires.

Vatican and the bomb

A group of scientists has given the Pope a tactless declaration on nuclear warfare.

The Pontifical Academy of Sciences is not an academy in the strict sense (its members are invited by the Vatican) but it seems well on the way to being pontifical. The statement put out at the end of September is thus a sombre warning of the dangers of nuclear war from which few will dissent, a stern proscription of steps that might trigger off such a war and an appeal to those concerned — politicians, scientists, religious leaders "and other custodians of moral principles" — to use their best influence for the avoidance of nuclear war. The statement is a remarkable document if only because of the distinction of those who have signed it — the list includes, for example, the presidents of the National Academy of Sciences of the United States (Dr Frank Press) and of the Royal Society (Sir Andrew Huxley), acting in their personal capacities. Its weakness is that the pursuit of agreement on a set of statements about nuclear war with which nobody can quarrel seems to have obscured the more immediate issues that must be dealt with.

Even the analysis of the problem of nuclear warfare in the published statement falls short in clarity of what the members of the group individually would suggest. Thus the statement says that "there appears to be a growing fatalistic acceptance that war is inevitable and that wars will be fought with nuclear weapons". Can that be so? At a time when there are more (and more hopeful) negotiations on the limitation of nuclear weapons under way than in the past three decades? Or when military authorities are seriously considering the possibility of defending Western Europe with conventional rather than nuclear weapons? It is true, of course, that political relations between East and West in Europe have deteriorated in the past year, for which reason it is all the more remarkable that the arms negotiators in Geneva are still talking to each other, however desultorily. Similarly, it does not follow, as the Pontifical Academy of Sciences suggests, that "proliferation of nuclear weapons to additional countries seriously increases the risk of nuclear war and could lead to nuclear terrorism". If anything, the argument should be turned around: as deep and apparently irreconcilable conflicts between sovereign powers become embittered (the Middle East?), the danger that one or other party will seek nuclear weapons increases. Proliferation (of which there has been mercifully little so far) is more likely to be a symptom than a cause.

The academy's recipe for action is similarly clouded. September's statement sanely asks that nations should renew their efforts to negotiate effective arms control agreements. The distinction of the participants should carry some weight, both in Geneva and in the continuing anxiety about the design of more effective measures for safeguarding the civil trade in nuclear power. But national governments are also asked "never to be the first to use nuclear weapons". Can that be seriously intended? For most of the signatories of the document must be fully aware that this is an extremely contentious issue. In Europe, the Soviet Union has repeatedly offered a "declaration of no first use", and its Western opponents have as steadfastly refused a deal. The reasons, moreover, are well understood. Either such a declaration means nothing, or it will favour that adversary in some future conflict which is weaker in conventional forces. To couple an appeal for more serious negotiations on arms control with a declaration calculated to lose the sympathy of many of those who will read the statement is tactless, to say the least.

What can be accomplished by such manifestos? There are two choices. Either a statement on some public issue can be so penetrating and original in its analysis of a problem, or so arresting in its description of it, that ordinary people will stop and think. Or it may so powerfully suggest new things to do that statesmen will be compelled by sheer logic to follow the course prescribed. This statement on nuclear warfare is, unhappily, neither one thing nor the other.

British research cake cut painfully

First public review reveals dissenting voice

Dissent among the five research councils has for the first time marred the customary gentility of the annual recommendations by the Advisory Board for the Research Councils (ABRC) on the British science budget. In what is meant as fighting talk, the Agricultural Research Council (ARC) says it "cannot associate" itself with the proposal that its own budget should take a declining share of the funds available beginning in the financial year 1984-85.

Ironically, this row has surfaced on the first occasion when the board's advice to the Secretary of State for Education and Science (Sir Keith Joseph) has been published. Sir Alec Merrison, the board's chairman, says that the row is unprecedented. The recommendations now made are provisional in that they will have to run the gauntlet of the British government's annual expenditure review, now under way. Sir Keith Joseph, the minister, has, however, already lopped £6 million from the proposed budgets of the Social Science Research Council (SSRC), asking that this sum should instead be used for recruiting young scientists to university posts, and has quickly fallen in with the recommendation that the British Museum (Natural History) should not spend in excess of £23 million to extend its exhibition space at South Kensington.

The recommended budgets for ARC imply a constant contribution from the science budget of £46 million in each year for the three years beginning next April, implying that the council will have to make good the unknown consequences of inflation by internal economies.

This parsimony towards ARC (and to a lesser extent towards the Natural Environment Research Council (NERC)) stems from the board's view that funds within the science budget should be diverted to other better causes — an increase in real terms of the funds available to the Science and Engineering Research Council (SERC) to support initiatives in fields such as information technology and for the provision of posts for younger scientists now frozen out of the research system.

Merrison explained last week that ARC had been singled out because of the large proportion (just over a half) of its budget channelled through the agriculture ministry to support commissioned applied research and because its links with universities are weaker than those of the other research councils.

But ARC has not yet lost hope. At a

recent meeting the council endorsed the formal dissent of its secretary, Dr Ralph Riley, from the ABRC recommendations, and in the next few weeks will be asking Sir Keith Joseph to take a broader view of national priorities in general and ARC's research enterprise in particular. If more funds are not found, ARC reckons that it will have to lose 300 staff over the next three years, of whom 200 will be scientists, and that in 1984-85 it will be spending £7 million out of a total budget of £107 million to meet the cost of staff pensions and severance pay.

ABRC's argument is guided by its explicit choice of the fields in which publicly supported civil science should be encouraged: biology (especially biotechnology), remote sensing, information technology, marine resources

and the neurosciences. But the board also says that its cutting of the cake this year reflects the view that the retreat from big science (experimental high-energy physics, space research) begun in the 1960s has gone far enough, and its general concern for the well-being of research and researchers in universities.

Running through the board's statement is its theme-song for 1982 — that funds spent on civil science, in universities or elsewhere, should be directed with industrial needs in mind. Its report says that it has "readily agreed" to help carry out a study (jointly with the Advisory Council on Applied Research and Development) whose terms of reference apparently leave no room for consideration of the snags that such collaboration may encounter. ●

Harvard accused of impropriety

Washington

A government audit has concluded that Harvard Medical School improperly charged \$1.7 million to federal research grants from 1975 to 1977 and failed to document a further \$29 million charged during the same period.

The findings by the Department of Health and Human Services (HHS), which runs the National Institutes of Health and the Public Health Service, were the latest salvos in the battle that has been waged between Harvard and the government during the five-year federal investigation of Harvard's management of grants. Last year, an HHS audit of Harvard's School of Public Health found \$2.1 million in improper charges by the university.

Asked whether Harvard would repay the sums in question, the university's director of auditing, Anthony Woodworth, said, "I very much doubt it". He said both audits are the subject of negotiations with HHS officials, and that they merely represent "recommendations" to the government, not orders for repayment.

Woodworth said that an independent audit by the accounting firm of Coopers and Lybrand of Harvard's management of grants in fiscal year 1978 "gave us a clean bill of health". He suggested that HHS's release of their recent findings was a public relations stunt.

The HHS audit specifically claims that the medical school improperly transferred \$1.6 million in charges from one project to another to cover cost overruns and to ensure that all available grant money was spent. The auditors said that some \$3 million was charged without adequate documentation, and that the validity of \$26 million that went to pay salaries could not be verified because of poor records.

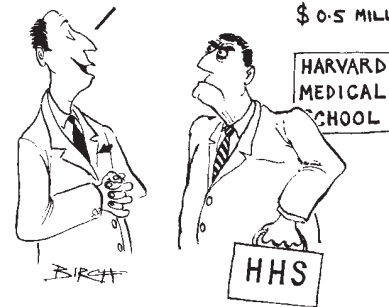
Harvard appears to be trying to suggest that the dispute is one of academic freedom

versus government meddling. Harvard and other universities have invoked academic freedom in resisting government efforts to require better accounting of how a faculty member divides his time between teaching and various research projects.

Harvard's attitude clearly annoys HHS. As one government official said, "What I find amazing is that we become the 'bad guys' for trying to reclaim the taxpayers' money".

Dr Delphis Goldberg, of the House Government Operations Committee panel that oversees HHS, said that the audits

"OF COURSE, YALE ONLY MANAGED A PALTRY \$0.5 MILLION."



found problems that went deeper than the dispute over effort reporting by faculty. At the School of Public Health, he said, "there has been a substantial diversion of moneys" from specific grants to totally unrelated research projects, even projects under different principal investigators.

The investigation of Harvard grew largely out of charges by Dr Phin Cohen, formerly an assistant professor of nutrition at the School of Public Health. In 1976, he accused the school of diverting funds from his NIH grant to other unrelated projects. A subsequent NIH audit confirmed his accusations — Harvard was required to return \$132,000 — and recommended a full-scale audit of Harvard research grants by HHS.

Stephen Budiansky

NSF budget for 1983

Spending lags behind inflation

Washington

With so much of the federal budget still in doubt for the fiscal year that started in October, the National Science Foundation (NSF) considers itself fortunate that both houses of Congress have approved an appropriation bill, despite their differences, and sent a recommendation to the President which he signed before the end of the old fiscal year.

Unfortunately, authorizing legislation — which spells out how the money should be spent — had been passed only by the House of Representatives, while because of a disagreement over who should look after NSF, two different Senate committees have worked up two different versions of NSF's authorization for fiscal year 1983. If the "lame-duck" Congress that returns later this month does not repair this situation, NSF may have to operate during the remainder of the fiscal year without detailed guidance — as it did last year.

The final amount appropriated was \$1,092 million, a little more than the President's request of \$1,078 million, but still close to the levels of the previous two years of \$1,003 and \$1,036 million respectively. With inflation, this means a net decrease.

The bulk of the money, \$1,060 million, will go to NSF's research and related activities. This includes \$14 million in funds taken out of other programmes which NSF can spend at its discretion.

Pre-college science education programmes, which the Administration tried to "zero" (a common verb in budget director David Stockman's vocabulary) survived with \$15 million. The total NSF science education budget will be \$30 million, the other \$15 million going to education fellowships requested by the Administration. The House of Representatives Appropriations Committee, more sensitive to the national "crisis" in science teaching, wanted \$25 million spent on pre-college education, and the Senate put in none: so they compromised.

The behavioural and social sciences were less successful in their attempts to resist Administration cutbacks. In 1982 the Administration tried to cut funds for these fields, but the social scientists fought back in Congress, and some of the money was restored. This year, the Administration requested \$36.6 million — and presumably that is what will be spent by NSF — but the funding is still below the 1980 level of \$47.9 million.

Among other decisions, both appropriations committees required that NSF should obtain their approval before spending money on the new ocean drilling vessel, *Glomar Explorer*. The conversion of the Central Intelligence Agency's vessel to a research ship has been very controversial and the committees wanted to see the results of the latest engineering studies. The \$14 million requested to operate the existing *Glomar Challenger* was reduced to \$12 million.

In September, just as researchers

planning to go to Antarctica for the summer season were getting ready, the Senate Appropriations Committee cut the US Antarctic programme's \$86.4 million budget request by \$15.3 million. This reduction would have cut the season's scientific programme by 60 to 70 per cent. In the end, only \$4 million was taken from the programme, leaving \$73.4 million for running costs and \$9 million for science. The cut will delay some construction planned for the base at McMurdo and mean later relief for the wintrine-over parties in August 1983.

International programmes at NSF continue their recent decline, from \$13.7 million in fiscal year 1980 to \$9.1 million in fiscal year 1983. The foundation has circumvented the Administration's and Congress's dislike of international science by funding international scientific travel out of the regular research funds. But because it puts international travel in competition with so many other things, it may accelerate the well-documented trend of declining US scientific travel abroad and fewer promising young US scientists taking fellowships overseas. **Deborah Shapley**

Research on ageing

US physicians come to life

Washington

Research on ageing has received some welcome attention in past weeks. First there was a meeting about recent research findings held at Rockefeller University, New York and sponsored by FIBER, the Fund for Investigative Biomedical Research, a relatively new private organization sponsored by medical philanthropist Mary Lasker and others. Then, in late October, the Institute of Medicine (IOM) devoted a day of its annual meeting to ageing research and the challenge to the US medical systems of the growing proportion of old people in the population (see *Nature* 26 August, p.779, and 2 September, p.2).

Both meetings were rich in suggestions from researchers in other fields for possible lines of inquiry into the mechanisms of ageing and into ways of prolonging cellular life or at least retarding decay. For example, at the FIBER meeting, Dr Allen R. Goldstein, chairman of the biochemistry department at the George Washington University School of Medical and Health Sciences, described the progress that has been made recently in understanding of the thymus: some recent work suggests that thymosin, which has been used to restore the immune response in cancer patients treated with chemotherapy, could also boost the immune response in older individuals. Joan Smith-Sonneborn of the University of Wyoming, a zoologist, described how paramecia can repair their own DNA through photoreactivation, and thus live a little longer.

Professor James Williamson, of the

department of geriatrics at the University of Edinburgh, talked about the social and financial problems posed by the large numbers of elderly people that will survive later in the century. Professor Williamson runs a geriatric service which consists of an interdisciplinary team of doctors and nurses. Patients referred to the service are visited at home within a day; only 30 per cent of them have to go to hospital and then usually for only a short time, or for regular short visits to give relief to their families. Such a service can reduce the number of old people in hospital geriatric wards by as much as 50 per cent.

A doctor from Los Angeles said that such a system would be impossible there, but Paul Beeson, editor of the American Geriatric Society's journal and formerly chairman of the department of medicine at Yale University medical school, disagreed. He said that most US doctors had their ideas of medicine shaped by the 1950s and 1960s, when funds were growing for biomedical research and specialities that could bring spectacular results were most sought after. Several leaders of US medicine have attacked the notion of geriatric medicine as not being medicine at all, he said, because it aims only to help people — and their families — and is more a social service than an investigative science.

Recommendations about how US medicine should address the problem of the nation's ageing population may emerge from a study that the Institute of Medicine and the National Academy of Sciences have set up and which is due to be completed next year. **Deborah Shapley**

Slaughter steps down

John B. Slaughter, director of the National Science Foundation, stepped down on 1 November in accordance with his decision several months ago to become chancellor of the University of Maryland College Park Campus. Since the Administration has not yet decided on a successor, Donald N. Langenberg, NSF's deputy director, is to become acting director of the foundation. Langenberg is likely to be a leading candidate for the position of director because of his extensive experience in various posts at the foundation.

Deborah Shapley

Madrid conference

Failing hopes for agreement

The review Conference on Security and Cooperation in Europe (CSCE), which reconvenes in Madrid next week, seems fated to be as inconclusive as the previous gathering in Belgrade (October 1977–March 1978).

The implementation of the Helsinki Accords has, if anything been hampered by increasing international tension, and, in particular, the imposition of martial law in Poland. So far there has been no agreement on the further development of the CSCE process. A possible draft statement (Document RM-39) drawn up in December 1981 by the non-aligned and neutral signatories of the Helsinki Final Act remains in abeyance, because the delegations from the United States and its Western allies have so far refused to discuss it while the situation in Poland shows no marked improvement.

The linking of negotiations on security and economic and scientific cooperation with those on human rights is one of the most sensitive issues in the Helsinki process. During the drafting of the Helsinki Final Act, the various proposals for cooperation were divided into three large filing baskets — security and arms control (Basket I), economic, scientific and technological cooperation (Basket II) and human rights (Basket III). The contention of the United States and some of its Western allies is that the signatory states must implement all three baskets. The Soviet Union and socialist bloc, however, prefer to treat the three baskets separately.

After the Belgrade review conference, it was decided to hive off scientific and technological cooperation — then considered the least contentious subjects — into a special “scientific forum” in Hamburg, held a few months before the main conference opened in Madrid.

The contents of Document RM-39 are for the most part acceptable in principle to all sides, although some of the “confidence building measures” associated with Basket I would doubtless need further detailed negotiation. (Thus the French proposal for an extension of the zone in which the Soviet Union should notify major troop movements back to the Urals has been countered by a Soviet demand that the NATO powers should notify major naval movements in the Atlantic.)

The Helsinki process, however, as the United States in quick to point out, is not a matter of legal wrangling but the creation of an atmosphere of detente. In the absence of such an atmosphere, they stated last March, it is impossible to discuss RM-39 or any other draft agreement. Since then the international climate has deteriorated even further. United States sanctions against the trans-Europe pipeline has evoked Soviet criticism that the United States is deliberately trying to sabotage detente and

urging that the pipeline is a success for Basket II.

The disappearance of the Helsinki Monitoring Movement in the Soviet Union (the last group, reduced by imprisonments to two members, dissolved itself last summer) is unlikely to improve the situation in Madrid. Nor is the recent renewed attack in the Soviet media on Anatolii Shcharanskii, a former member of the movement, now serving a 13-year sentence for human rights activities which

UK university mergers

No links for UMIST and Salford

A combination of institutional loyalty and academic snobbery seems to have frustrated one of the most promising of amalgamations between British universities — a much canvassed proposal that the University of Salford and the University of Manchester Institute of Science and Technology (UMIST) should join forces. Two weeks ago, the UMIST governing body (its council) decided that, among several possible options, it would prefer to forge stronger links with its parent university, the University of Manchester.

Both partners in this abortive marriage have just lived through a hard year. Salford has been adjusting to the prospect of a sharply reduced subvention from the University Grants Committee (UGC), discovering that the extension of the transitional period has not allowed it to avoid painful retrenchment. UMIST, three miles away, has spent the past year wrestling with the problem of whether its principal, Professor R.N. Haszeldine, should remain in the post to which he was appointed in 1975. The cost of modernizing the principal's residence was a contentious issue. Professor Haszeldine, distinguished for his research on fluorine chemistry, has retired early with effect from the end of this year.

Almost immediately that issue had been resolved, UMIST found the local newspapers full of a proposal by Mr Fred Sylvester, one of Manchester's Members of Parliament, that Salford and UMIST should agree to merge. The logic of such an arrangement seems irrefutable; both institutions concentrate on science and technology (but have important outside interests, in politics and modern languages respectively, for example) while they are physically separated by only three miles.

The University of Salford now says it is disappointed that the prospect of a merger between the two institutions has disappeared. People at UMIST, on the other hand, profess themselves offended that they first heard of the proposed merger from newspaper reports, and that there has never been a formal approach from

the courts found treasonable. Nor is the arrest, in Poland, of the mathematician Dr Zbigniew Romaszewski, who in 1980 compiled and sent to Madrid a report on human rights abuses in Poland.

Under these conditions, it is uncertain whether any useful discussions can be expected from the forthcoming session. If the United States once again refuses to discuss Document RM-39, there would be little point in continuing the session. Sooner or later, if no final statement is agreed, the Madrid Conference will go the way of its predecessor in Belgrade; it will have to agree to disagree, and set a place and date for a new Conference. **Vera Rich**

Salford.

The preferred option of the UMIST court to strengthen links with the University of Manchester, is intended to build on the institute's constitution as the Faculty of Technology of the university. The university awards degrees to graduates of the institute, but otherwise relations between the two over the past thirty years have been close or distant according to the personalities of those in charge. UMIST now cites as an example of the collaboration foreseen with its parent institution the occupation of the same building by two departments of metallurgy, its own and that of the university, saying that it has asked for further discussion of such cooperation.



The University of Salford — to remain alone

By common consent, Salford has survived the hardships of the past two years with flair, largely because of the success as vice-chancellor of Dr J. M. Ashworth, the most centrally placed (but not the most senior) of the British government's scientific advisers until the end of 1980. One Salford academic said this week that Ashworth has given Salford such a sense that it has a future that hope is now pinned on the belief that UGC will next year relent.

Both institutions are on course in their reduction of student numbers, while each of them has increased the intake of overseas students in the current academic year, Salford by 46 per cent and UMIST by half as much. ●

US medical schools

Counting social costs

Washington

There is a story making the rounds of college administrators in which a university president is greeted by the Devil at the gates of Hell with an offer of the presidency of the local university. Delighted, the president immediately accepts, remarking that he had expected far worse treatment. "Yes", Satan replies with a glint in his eye, "but our university has *two* medical schools."

If that is indeed the American university president's personal vision of hell, then some recent changes in government health-care financing may be a foretaste. Stanford University president Donald Kennedy, for one, has left no doubt that the federal government is taking the part of the Devil in imposing limitations on the reimbursement to hospitals for treating poor and elderly patients covered by government programmes.

Kennedy's views are shared widely among university-affiliated teaching hospitals, which see themselves as innocent victims of government's efforts to control rocketing health-care costs, and in particular those costs borne by the government itself.

In the past ten years, federal spending on Medicare (which covers the elderly through the social security system) climbed from \$8,800 million to more than \$44,000 million; spending for Medicaid (which covers the poor through the welfare system) rose from \$4,700 million to more than \$17,000 million. Even before the coming of Reaganomics, these seemingly uncontrollable expenditures were a matter of concern within Congress and the executive branch.

But to the universities, even these modest cuts have been a source of worry. According to a recent report prepared by the Association of Academic Health Centers, Medicare and Medicaid payments to university-affiliated teaching hospitals allowed them to expand their faculties and their educational programmes during the late 1960s and 1970s. This dependence has made them acutely sensitive to any efforts to limit reimbursements.

So, with some new and much tougher limitations in effect from 1 October, the complaints from universities have grown louder. Kennedy, in his Harvard speech, called the government policy "disastrous". The crux of the matter, he said, is that the government is no longer reimbursing hospitals for the full cost of Medicare and Medicaid patients. This forces the hospitals to raise their charges to private patients, and puts a disproportionate burden on teaching hospitals, which receive a high proportion of difficult and charity cases. Furthermore, the higher costs to private patients at teaching hospitals means that more of the routine —

and profitable — business is going to county and private hospitals.

The shifting of costs from Medicare and Medicaid patients to private patients has been well-documented by the insurance industry, which ends up paying more. According to Neil Swan of the Health Insurance Association of America, the government shortfall will amount to \$5,800 million this year. Kennedy said that charges to private patients at Stanford University Hospital could be reduced overnight by 14 per cent if Medicare and Medicaid reimbursements covered true costs.

And data from the Association of American Medical Colleges (AAMC) seem to support the universities' contention that they carry a greater burden than most. A comparison of the 327 member hospitals in AAMC with the 5,503 non-AAMC hospitals in the American Hospital Association found that although the teaching hospitals accounted for 26 per cent of total net patient revenue, they carried 35 per cent of bad debts and 47 per cent of the charity write-offs.

The new rules impose, for the first time, a limit on the annual increase in all hospital costs paid for by Medicare and Medicaid — a limit that will widen the gap between government reimbursements and the true costs of treating government-supported patients. The increase for 1983 has been set at 7.9 per cent; hospitals whose costs rise more than that will have to bear part of the excess themselves. The new rules also require that among groupings of similar hospitals, no-one can run costs greater than 120 per cent of the group's average. (That limit will drop to 115 per cent in 1984 and 110 per cent in 1985.) Together, these measures are expected to save \$480 million in 1983 and \$3,770 million by 1985.

The universities' complaints fall on less-than-sympathetic ears at the Senate Finance Committee, which, at the instigation of Senator Robert Dole (Republican, Kansas), wrote the legislation ordering the new rules. One staff member said "you hear that griping all the time", but that the rules contain several special provisions that favour the universities. Most important is exemption from the new limitations for direct educational costs of teaching hospitals, for example, interns' and residents' salaries.

The new rules do contain, however, a direct blow to one bountiful source of funds for the universities under Medicare and Medicaid: faculty practice fees. These are fees paid for the professional time of faculty physicians who treat Medicare and Medicaid patients. The faculty physicians turn these reimbursements over to the university, which uses them partly to supplement faculty salaries and partly for "dean's funds" that pay educational expenses, such as equipment purchases.

Under the new rules, faculty practice fees cannot exceed the faculty member's salary.

The situation is complicated by a proposal made public earlier this month by Richard Schweiker, Secretary of Health and Human Services, that would completely overhaul the method of government payment under Medicare and Medicaid. This plan, known as "prospective reimbursement", would set reimbursement rates in advance. The most serious reservation is that, like the 1 October rules, it does nothing about the problem of the cost-shift to private patients. The insurance industry, which is particularly eager for action, points to successful state programmes (in Maryland and New Jersey, and soon in Massachusetts and New York) that combine prospective reimbursement with state regulation of charges to all patients.

Stephen Budiansky

Lead in petrol

Europe too soft?

Brussels

A new attempt is being made to revive the debate about lead safety levels by European consumer and environmental groups. Despite numerous directives defining safety levels in everything from the workplace to shellfish, EEC is now being accused of setting lower safety levels than either the United States or Japan. Calls for a revision of European standards of lead in petrol were made last week by European associations of consumer groups (BEUC) and environmental groups (EEB).

Since 1978, an EEC directive has permitted lead in petrol levels of a maximum of 0.4 grammes per litre and a minimum of 0.15 grammes per litre. The United Kingdom's decision to enforce the lower level from 1985 onwards aroused considerable controversy earlier this year because of scientific evidence that even low concentrations of lead in air could have harmful effects on the nervous system of critical groups such as children and pregnant women.

A new campaign is being launched to persuade the European Commission to revise the directive, and to introduce lead-free petrol throughout the member states. No member state is at present allowed to do this and pressure is building up from inside and outside the European Community. Germany and Denmark are in favour and Sweden, Norway and Switzerland are threatening to take unilateral action. The consumer and environmental lobbyists argue that if the two other main car-producing countries are convinced of the desirability of lead-free petrol, so should be the Community. Japan started as early as 1968 and the United States began to phase out leaded petrol in 1974, although small refineries are still allowed to produce leaded petrol.

Experience in both Japan and the United

States suggests that there are few penalties for introducing lead-free petrol. The added cost to the consumer in the first two or three years is probably no more than £0.8 pence a gallon, according to the UK Ministry of Transport, while the lobbyists claim that studies in the United States and Canada prove that there are definite savings in vehicle maintenance costs of perhaps \$0.05 a US gallon. In addition, European car manufacturers already make cars for the US and Japanese markets designed to run on lead-free petrol.

But industry is unlikely to be happy about being asked to make new investments when there is already a massive over-capacity in European refineries and the car industry is faced with a prolonged recession.

Their defence rests mainly on placing doubt on the scientific evidence of low concentrations of lead in air causing damage. While EEB and BEUC admit there is unlikely ever to be exact correlations between, for example a reduction of lead air concentration and the number of mentally sub-normal children in the EEC, there is enough evidence to warrant action.

The Isotopic Lead Experiment being sponsored by the Commission in Turin and Piedmont indicates that at least 30 per cent of the lead in blood comes from petrol, although there are, of course, plenty of other sources such as paint or plumbing. In the experiment, lead from Broken Hill Mine in Australia with an easily recognizable mix of isotopes 206 and 207 was put in the petrol sold in the area between 1977 and 1979 thus enabling the source of the lead in blood to be immediately identified. The full report of the research has yet to be released on the grounds that there has been some difficulty in interpreting the data, but there are now rumours that the real reason is the alarming nature of the conclusions.

The arguments will undoubtedly continue, though, on what constitutes a safe level of exposure. This year EEC approved a directive for a maximum blood lead level of 60 microgrammes per decilitre in the workplace despite calls from the European Parliament and some member states for more stringent standards.

The European Commission wants to review the blood lead reference levels which are the criteria for assessing the need to take remedial action. The reference levels are a maximum of 20 microgrammes per decilitre of blood for 50 per cent of the population, 30 for 90 per cent and 35 for 98 per cent of the population.

The Lawther Report in the United Kingdom, published in 1980, asserted that there was no convincing evidence of deleterious effects below the 35 microgrammes per decilitre level. Yet an American study by Davidow *et al.* in New York in 1982 on the influence of lead on the production of haemoglobin suggests that the blood lead level threshold below which children's haemoglobin production is not affected is 15–18 microgrammes per decilitre.

Jasper Becker

Blue skies all around

The pace of industrial investment in British academic research seems to be quickening. After several years of niggardly treatment at the hands of the British taxpayer, British academics appear to be more willing than ever that their research should be supported by industrial money. This is the spirit in which British Petroleum's Venture Research Unit, formed by BP Limited two years ago to support what is called "blue skies research", decided to invest £500,000 in four novel academic projects, three in Britain and one in Austin, Texas.

The four contracts agreed upon last week (but yet to be negotiated with the intended recipients) illustrate what BP means by blue skies research. One, with the University of Aberdeen, is an exploratory project further to investigate the possibility that L-phase bacteria may be able to transfer genetic material directly into the cells of plants.

The venture unit is also offering research contracts to the University of Sheffield to investigate the application of control engineering methods to the study of photosynthesis and to the University of Dundee for an investigation of the apparently superior properties of amorphous silicon as a material for semiconductors. The proposed contract with the University of Texas, Austin, is for the development of the work of Dr Jess Kimble on optically bistable devices, possible components in future computers.

Some of the score or so of projects so far backed are relevant to the immediate needs of BP, which maintains its own much larger in-house research

programme for that purpose. According to Dr D.W. Braben, the manager of the venture unit, the objective is to find people who are preoccupied with some unusual problem and who appear to have the ingenuity to solve it. Many of the contracts so far signed are concerned with the mathematics of complex processes, with computer applications and with the behaviour of microorganisms. Under the usual arrangement with an academic group, publication is allowed until commercial exploitation is foreseen.

The University of Sheffield is also the partner in another industrial venture announced this week — the founding of a company (Plant Science Ltd) to exploit several applications of plant science developed at the university's Institute of Biotechnology, founded with a grant from the Wolfson Foundation in 1978 and converted into an independent unit (with a further grant of £360,000) in March 1981.

The new company has been started with a capital contribution from TDC, described as "the high-technology arm" of Finance for Industry, a kind of merchant bank supported by various clearing banks. Among the objectives of Plant Science Ltd will be the development of plant varieties yielding substantial quantities of chemicals (such as steroids) and of processes for their extraction. The technical director of the new company is Dr Michael Fowler, from the university institute, who with Dr Anthony Jubb (recruited as managing director from ICI Ltd) will share part of the equity with TDC and the university. ●

UK research councils

Spend, spend, spending

Next to the cost of defence and of the police, the British science budget stands out in British public expenditure by having increased in the past three years even more quickly than the rate of inflation. Sir Alec Merrison, chairman of the Advisory Board for the Research Councils, said last week that "you cannot stay in that position without paying a price". Part of the price may be the publication last week of the board's advice on how to cut the cake (£507 million in 1983–84, 1.2 per cent more than needed to compensate for inflation).

The board has the following comments to make on the cases put to it by its pensioners, five research councils, the Royal Society and a museum.

The Agricultural Research Council (ARC) plans to maintain and even increase support for university research, especially in biotechnology, reproductive endocrinology, plant growth hormones and food process engineering. A new computer centre is planned. If there were more

money (there is not) it would be spent on the genetic manipulation of animals, vaccine production and the micro-propagation of plants.

The board says that ARC should press ahead with its priority projects and should seek outside sources of financial support for some of this but recommends a constant cash contribution from the science budget in the next three years of £46 million. ARC's total income would increase from £95.9 million in the present financial year to £107 million in 1985–86 on the assumption that the agriculture ministry would pay more for commissioned research.

The Medical Research Council (MRC) plans a scheme to rescue biomedical research groups left stranded by university economics up to a total of 20 groups at a cost of £50,000 each in each of the next five years, and a modest increase of graduate student support. The council promises "major restructuring" at the National

Institute for Medical Research and at its Radiobiology Unit, and clinical trials in such fields as cancer treatment, NMR imaging and multiple sclerosis.

The board recommends that MRC should "review" its participation in the European Molecular Biology Laboratory (Heidelberg), warns the council not to spend research funds in neglectful universities and recommends that the council should have a constant 22.5 per cent of the science budget, amounting to £113 million next year which will be increased by about £5 million from other sources.

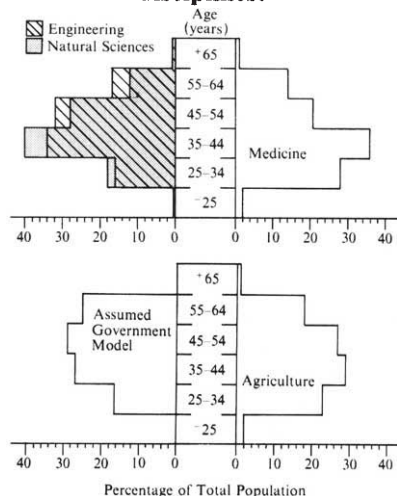
The Natural Environment Research Council (NERC) plans to support more university research and graduate students, and to continue diverting resources towards remote sensing. Participation in the international ocean drilling programme beyond 1983 is uncertain, given the likely 50 per cent increase of cost.

The board recommends a share of the science budget rising from £58 million in 1983-84 to £63 million in 1985-86, including a constant £0.4 million for ocean drilling. At the end of the period, NERC will be £1.7 million worse off than now, but will earn £29 million from commissioned research.

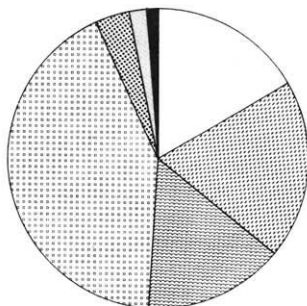
The Science and Engineering Research Council (SERC) plans to increase support for biotechnology and civil engineering, to cut back in marine technology and polymer engineering, to reduce its payroll by 1 per cent in each of the next three years and to divert resources of two "major new programmes" — information technology and space research, perhaps at a total cost of £50 million over three years.

The board accepts that the time has come to halt the decline of "big science", recommends that the council should have an extra £22.5 million over three years to support basic research in information technology but that it should find the extra needed from its own resources. The board says that the council's budget should in-

Age distribution in British academic disciplines.



Data from the ABRC report showing age distribution in various disciplines together with the steady-state distribution (bottom, left).



- Agricultural Research Council
- ▨ Medical Research Council
- ▤ Natural Environment Research Council
- ▧ Science and Engineering Research Council
- ▩ Social Science Research Council
- British Museum (Natural History)
- Royal Society

Relative income of research councils, etc., in 1982-83. Figures include allocations from the science budget, commissioned research and other receipts. Total income is £579 million. (Source: *The Science Budget, a Forward Look*, the Advisory Board for the Research Councils.)

crease from £234 million now to £250 million next year and £278 million in three years, to which is added about £10 million in other income.

The Social Science Research Council (SSRC) pleads for a restoration of the funds cut from its budget in recent years, promising new research training schemes and boasting of a smoother organization. The board's recommendation accepts this

case, suggesting a share of the science budget rising from £23.3 million next year to £25.5 million after three years. These are the sums from which the Secretary of State for Education and Science has subtracted in advance a total of £6 million. Sir Alec Merrison said last week that his board had no hard feelings on the subject, saying that the minister was the one with ultimate responsibility.

The British Museum (Natural History) asked the board for a general increase of funds to strengthen its general programme, and has been repaid with the recommendation that the proposed extension of the museum's exhibition space (at a cost of between £23 and £29 million) should be abandoned. The board says that the project, "however worthy, has no direct connection with scientific research".

The Royal Society plans to spend £1 million a year on a scheme for 100 new research fellowships and asks for half that amount to support exchanges with the United States, while promising stronger collaboration with Japan. (The society laments that the Australian government is unwilling to help with the cost of the present Anglo-Australian exchange scheme.)

The board accepts the case that money should be spent on people rather than on instrumentation and project grants, and recommends an increase of £0.5 million in the society's budget to £5.0 million next year rising to £5.8 million in 1985-86. The society's total annual income is nearly £2 million greater. ●

Environmental research at risk

Oceanography and forestry departments in British universities may be threatened by university efforts to trim spending in line with government cuts, according to Sir Hermann Bondi, chairman of the UK Natural Environment Research Council (NERC).

"It is of the utmost importance" writes Sir Hermann in the 1982 annual NERC report (HMSO, £4.25), published last week "that [the universities] manage their affairs in their present difficulties so as not to weaken their research capabilities . . .". Small fields, common to only a few universities, are particularly vulnerable, believes Sir Hermann — and he is clearly itching to tell the universities what to do without quite daring to. "Our ability to intervene and to help is clearly very limited" he writes. NERC has offered to help relocate departments from one university to another and to allow a degree of rationalization of resources in the universities — "but we must wait for their initiative . . .".

Sir Hermann has also had to wait for the initiative of certain government departments, not named in the report but certainly including the Department of the Environment, which have been very slow to define a long-term contract research

policy. "Some of our customers find talking to us a little strange" said Sir Hermann last week. The strangeness is legal: advice has been given that a verbal agreement is as binding as a written one, with the result that "certain departments" have refused to discuss possible future funding or policy. This has led to considerable difficulties at NERC, which must assume that it always has the correct programme of basic research to provide expertise for any applied contracts that may be placed with it.

The Rothschild principle, where a portion of a research council's cash is obtained by contract from a customer department, was still "fundamentally right" said Sir Hermann. However, being right in principle was not sufficient — the department itself had to have great scientific competence. One of Rothschild's objectives had been to create that competence in the form of chief scientists' divisions — but in some departments the chief scientists had been pushed to one side. "They must be at the centre of decision-making" said Sir Hermann. The scientific attitude of the chief scientist should "permeate the whole department". **Robert Walgate**

US geological research

Asset-stripping fear at survey

Washington

It has been a long, hot summer for the US Geological Survey (USGS) the United States' oldest basic research agency. A chain of decisions by the controversial US Secretary of the Interior, James Watt — that stem from a scandal in one part of the survey dating back to 1959 — had led by 1 October to the departure of approximately 2,400 people, including the entire Conservation Division and a third of USGS's marine geology research, to a new agency in charge of leasing federal lands, the Minerals Management Service (MMS).

One survey scientist describes his organization now as "a car going down the street and someone takes a wheel off". By October, Dallas Peck, the director of the survey who is one of its career scientists, had adroitly negotiated with Watt, who is his boss, to minimize the damage to the survey as a research institution, and cut back the "raid" of marine geology from Watt's original plans.

The loss of only a third of the marine geology group was considered a triumph of sorts by survey scientists who, over the summer, had been comparing departure plans. Morale was described as good, even though rumours were still circulating that Watt might now also ask the coal and marine scientists at the survey to come over to the politically-led MMS.

USGS conducts basic geological research, but since the nineteenth century it has also had some applied missions. The most important of these, in recent years, has been to make geological assessments of federal and Indian lands, both in the "lower 48" states and in Alaska, and of the enormous US continental shelf. These assessments are used by another agency of the Interior Department, the Bureau of Land Management, to second-guess oil and minerals company bids to explore and develop these lands, and for devising overall policies on which lands should be developed. USGS had the job of determining the "fair market value" of the lands and of specific tracts, to make sure the government made the corporations offer high enough royalties to flow to the federal and state coffers.

Both these tasks have been intensely controversial, and pitted the Interior Department, the Bureau of Land Management, the government, the companies, the environmentalists and the Indian tribes against each other in a fashion that makes the development of the North Sea and northern Canada look orderly by comparison.

The misnamed Conservation Division of USGS had the job of regulation, including royalty collection. Geologists in the Geologic Division, which includes the marine geologists, dealt with resource assessment and determining fair market

value.

Over the years, many lands were leased for oil and mineral development in the Western states, and after the discovery of oil at Prudhoe Bay, Alaska, and the 1973 oil embargo, the leasing of lands grew, as did royalties. The Conservation Division did not have the manpower to handle these tasks. When Watt took office in early 1981, he unearthed a series of old General Accounting Office reports dating back to 1959, claiming USGS had failed to collect enough money.

Watt appointed a commission, headed by a businessman, David F. Linowes, to look into the loss of revenues. In January 1982, acting on the Linowes Commission report, he decided to transfer the handling of royalties to a new agency, the Minerals Management Service. But he went further than the Linowes commission and moved the entire Conservation Division — approximately 2,200 people — to make the bulk of the new MMS. MMS would handle the assessment, leasing and royalties collection for offshore lands, as well as the assessment, monitoring and royalties collection of onshore lands. Its powers over the onshore lands may yet be increased.

The transfer of the Conservation Division in January made big cuts in USGS, which had 10,000 full-time positions, and a budget proposed at \$516 million. But it was considered by many to be an appropriate streamlining of the offshore leasing process, which had hitherto required many different agencies (or as one source says "35 people in the room with Watt") to coordinate in order to issue a single lease.

Far more controversial was another directive — a surprise to USGS and, apparently, to its director, Peck — that Watt sent in May asking that the Marine Geology Division, which makes offshore resource assessments, should also move over to MMS. Although the marine geology work consists of only 442 full-time positions and funding of \$22 million, the division considers itself part of the scientific heart of the survey. Everyone was upset at the thought of these people, who pride themselves on being as good as university professors, working for the political MMS, which is run by Harold E. Doley Jr, a 35-year-old black Louisiana stockbroker who had helped get southern black votes for Reagan in 1980.

"All of a sudden, MMS would acquire all those ships and photographic equipment and scientists who were more senior than most of the personnel of MMS" comments one survey scientist — indicating the shock caused by Watt's memorandum.

Peck, however (who could not be reached for interview), took the blow in his

stride, and argued that the agency was stronger. To his staff, in May, he said that "the focus of our activities is now even more clearly upon the scientific and technical aspects of our mission . . . It is now our responsibility to assure that we continue to earn the confidence which the secretary has placed in us. This will require that we look carefully at everything we are doing to ensure that our actions are responsible to the department's goals."

Peck negotiated with his bosses at the Interior Department all summer, and by the summer's end had reached an agreement by which only those marine geologists who really were involved in resource assessment and applied jobs would leave the survey for MMS. Finally, only 147 positions and \$8 million left the programme. According to an earlier plan, marine geology was merged with energy, within the Geologic Division.



Harold Doley Jr — MMS director

Watt explained to a group of visitors to the White House a few weeks ago his belief that the government must facilitate the orderly development of the country's mineral and energy resources to avoid a "panic" rush to develop them in the twenty-first century.

The controversy, and continuing fear of another Watt raid on other researchers, has been high drama for the Geological Survey, which enjoyed a carefully nurtured respectable obscurity around Washington. But there are more important issues at stake, namely whether assessment should be carried out within a scientific agency if it is to preserve its integrity.

One commonly expressed fear is that MMS will be so anxious to respond to Watt's wishes that it will undervalue the tracts and not require high enough bids from industry — thus depriving federal and state governments of revenue. It will be some time before it can be determined if this major shift in science policy is a mistake, and loses the taxpayer money. If it does, it will be an ironic outcome for a process that began by trying to recoup funds for the public purse.

Deborah Shapley

CORRESPONDENCE

EMBL's databank

SIR — We have had several comments concerning the recent letter from A. Slocum (*Nature* 7 October, p.482) describing a commercial timesharing service offered by Intelligenetics, Inc.

Intelligenetics makes available extensive software and computing capacity together with two nucleic acid sequence databases, including the one maintained by our group at the European Molecular Biology Laboratory (EMBL).

We wish to point out that the EMBL sequence data library has been sent to more than one thousand organizations, both academic and commercial. The data collection is supplied to everyone on the same basis: no restriction is made on its use or redistribution. Intelligenetics is free to offer access to the data in the context of their computing system, as is anyone else who may wish to do so. No-one has special or exclusive licence to the data: they remain freely available to any individual or organization directly from us.

GREG HAMM
KURT STUEBER
GRAHAM CAMERON

EMBL Nucleotide Sequence Data Library,
Heidelberg, FRG

Keep the index

SIR — Having read King's critical comments (*Nature* 30 September, p.390), I would like to express my approval of your biotechnology stock index.

The success of these corporations will reflect the ability of biotechnology to find a place in public and private business. There are many more biotechnology corporations, some without public stock offerings, that are just as important. It is not necessary to list every firm to achieve a general overview, as *Nature* has done so well.

I hope *Nature* will not hesitate to add or drop corporations from the index in keeping with trends in this field.

SQUIZZLE PLEKAVICH

Arlington, Massachusetts, USA

Preventing wars

SIR — I was pleased to read that J.R. Skoyles (*Nature* 7 October, p.482) approves of the proposals for a "comprehensive ban on all methods and means of warfare specifically designed to kill or injure by inducing human disease".

I feel that the criticisms with which he continues his letter are based on a misunderstanding of the second part of my letter (*Nature* 9 September, p.102). It was concerned only with the disarmament side of the disarmament/international security relationship¹, as is the UN draft Comprehensive Programme of Disarmament (CPD). The draft was referred by the UN Second Special Session on Disarmament back to the Geneva Committee on Disarmament for

them to bring a revised version to the 1983 regular Session of the General Assembly. We believe that the content of the draft should be a matter for informed public discussion and comment, and wish to contribute to encouraging this.

The approach in the existing draft CPD is across-the-board disarmament in three (or four) stages. The arguments against this approach are: a 50-year history of political failure; the unrealistic requirement diplomatically and technically for simultaneous negotiations and verifications in all five categories of disarmament measures of Stage I; requiring the final stage of nuclear disarmament to coincide with the final stage of conventional disarmament, despite uncertainty on whether world society can achieve the latter; and the preclusion of a selective comprehensive treaty banning non-nuclear weapons of mass destruction and all disease-inducing methods of warfare, which is the only immediately feasible treaty of comprehensive disarmament².

The specific measures mentioned in the second part of my letter were only suggestions of what hopefully might follow the conclusion of such a treaty, if the disarmament diplomats could be persuaded to replace their "disarmament in layers" by a more meaningful approach.

JEFFREY SEGALL

London NW2, UK

1. UN. A/36/597, 19 November 1981.
2. Meeting of Professions for World Disarmament and Development, 2 Oct. 1982.

The fetal soul

SIR — I beg leave to disagree with the comment in your leading article that "gastrulation is the latest point at which twinning can occur and thus the first point at which one body can have a unique soul" (*Nature* 7 October, p.475).

In one of the most solemn declarations of the Catholic Church Pope Pius IX in 1854 declared:

The blessed Virgin Mary was, in the first instant of her conception, through the merits of Jesus Christ the saviour of the human race, by a singular grace and privilege of God almighty, preserved immune from every stain of original sin.

It is thus certain for Catholics that the soul enters the fetus at conception. If the physical fetus divides, God is as capable of creating a second soul as He was of creating the first — thus both bodies will have immortal souls, each of which will eventually spend eternity in a state of friendship with God (Heaven) or enmity with God (Hell).

DAVE PARRY

Glasgow G12, UK

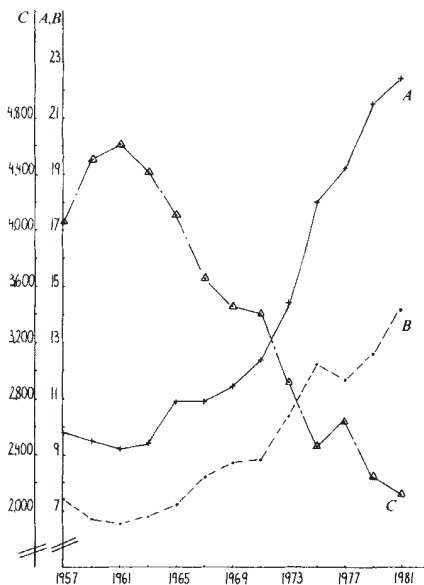
Costly journals

SIR — Robert Campbell was right to point out in an introduction to *New Journals Review* (*Nature* 7 October, p.49) that falling circulations do bring sharp increases in journal prices, thus causing a further drop in subscription levels, and further price increases.

It is a problem we have been interested in for some time now¹ and have studied quantitatively in relation to physics periodicals. The problem facing libraries is indeed difficult. Taking our sample of 160 journals as an example, if a library wanted to keep up with both the growth in the number of core physics journals and their rising prices, it would have needed to increase its subscription budget by a factor of 30 since 1957.

We have shown elsewhere³ that most of the price increases can be attributed to falls in circulations, but we have also demonstrated that, for the last few years, journal prices have been rising in anticipation of even greater circulation falls (see figure).

The timing of this change corresponds to a fall in the size of the market for physics journals. Unless some drastic steps are taken to reverse this trend, we predict that, as far as physics journals are concerned, the circulation



Graph showing the impact of the fall in circulation on journal prices in a sample of 160 core physics journals. Scale A, the actual price per standard volume, in constant (1957) dollars; B, the "expected" price per standard volume, in constant (1957) dollars; C, the average circulation per title for journals in the sample. A standard volume contains 500 standard pages¹. The "expected" price is calculated by taking into account increases in publishing costs and falls in circulation². Note the increasing divergence of the two price curves.

will continue to fall³. By the year 2000, the average subscription level of a core physics journal will be approximately 900, a fivefold drop since the late 1950s. Prices will, of course, continue to rise.

S. CHOMET
E. POLLITZER

Department of Physics,
Kings College,
London WC2, UK

1. Chomet, S. & Nejman, E. *Nature* 279, 188 (1979).
2. Nejman, E. thesis, Univ. London (1981).
3. Chomet, S. & Nejman, E. *J. Research Commun. Stud.* 3, 185 (1981).

NEWS AND VIEWS

How large is the Universe?

Estimates of the Hubble constant have fluctuated frequently in recent decades, usually between 50 and 100 km s⁻¹ per megaparsec. There is still much to be done to fix the value.

Sir Hermann Bondi once caused great consternation among an audience of physicists by declaring that because the essential parameters of the Universe were then (in the early 1950s) so poorly known, and so likely to be wrong, he proposed to ignore what passed for numerical data in a lecture on cosmology. Since then, matters have to some extent improved. Since 1974, for example, a monumental study by Sandage and Tammann (see, for example, Sandage, A.R. and Tammann, G.A., *Astrophys. J.* **194**, 559; 1974) has seemed to provide a reasonably secure basis for estimating the distance scale of the Universe. To be sure, there has been a constant rumble of discontent with the resulting estimates, so much so that most articles dealing with distant phenomena have prudently included a statement of which estimate of Hubble's constant has been used in the interpretation of data. Now the issue will have been thrown into the melting-pot again with the publication by David A. Hanes of the Anglo-Australian Telescope of an argument that the extragalactic distance scale has been overestimated by 50 per cent or so (Hanes, D.A. *Mon. Not. R. astr. Soc.* **201**, 145–170; 1982).

Nobody is to blame. The problem of laying down a distance scale within the local galaxy, which occupied the best part of a century, was a sufficient illustration of the difficulties of such exercises. As things turned out, it may be counted simply luck that there is a class of stars, the Cepheid variables, whose period of variation is a function of their luminosity, and which can thus be used as milestones within the local galaxy. Experience has so far revealed no comparably convenient method for the measurement of extragalactic distances. Instead, people like Sandage have been forced to rely on much less sharp correlations between the characteristics of some distant galaxy and its probable luminosity. But only in the past decade or so has it become clear that the statistical distance yardsticks now developed are undermined at least to some degree by the likelihood that the galaxy from which all observations are made (our own), and the Local Group of galaxies to which it belongs, are probably moving towards the centre of the Virgo cluster, probably under the influence of gravitational attraction.

What Hanes has now done is to work step by step through the analysis by which Sandage and Tammann set up their distance scale ten years ago. There is no dispute about the distance to the galaxies belonging to the Local Group — the Magellanic Clouds and the Andromeda Nebula (M33) for example — for Cepheid variables are still recognizable there and can be used as more or less certain yardsticks. Moreover, these galaxies of the Local Group serve as proving grounds for other kinds of distance yardsticks — other kinds of variable stars, red giants and the like. Hanes now points out that the crucial step in the Sandage analysis is the extension of this kind of argument to the next nearest group of galaxies, that centred on the object known as NGC2403, for this is the most distant galaxy in which Cepheid variables can still be recognized as such. The essential difficulty is that light from these variable stars may be obscured to an unknown extent by the material of which the galaxy consists, thus reducing the perceived luminosity of the objects. Hanes follows others in arguing that too little allowance has been made for obscuration, with the result that the estimate of the distance of even NGC2403 has been overestimated.

The trouble with this conclusion, forcefully advocated by Hanes, is that it applies to the other tests used by those who set out to establish extragalactic yardsticks. One of the most obvious of these is based on the assumption that the brightest (and thus the most easily distinguishable) stars in a distant galaxy are likely to be indicators of the distance of that object. The more distant a galaxy, the fainter its brightest blue stars should seem to be. The obvious pitfall is that the brightest stars in a galaxy, lying as they must on the extreme tail of some gaussian distribution, can only be indicators of distance in some statistical sense. Mercifully, extragalactic yardstick-makers are reconciled to that. Hanes's particular contribution to this discussion is to show that the brightest star criterion depends on a piece of circular reasoning. Much the same is true, says Hanes, of the other criterion used as a distance marker by Sandage and Tammann — the notion that the size of molecular hydrogen clouds associated with galaxies should statistically represent their distance from the local galaxy (which implicitly supposes that while galaxies and their associated molecular hydrogen clouds may differ from each other in their extent, the range of this variation is not *too* great). Hanes finds himself putting the reasonable proposition that the visual appearance of a distant galaxy, which is a guide to its intrinsic luminosity in some poorly known statistical sense, may be a better criterion than any other used so far. The proposition is reasonable because it depends on integrated light intensity, not the luminosity of exceptional objects.

Hanes thus comes to the conclusion that the Hubble constant is more probably larger than smaller — more like 100 km s⁻¹ per megaparsec than half as much. Indeed, he neatly splits the difference between the estimate of Hubble's constant published a decade ago and that of 100 km s⁻¹ per megaparsec: if the measured redshifts of the Virgo cluster galaxies are taken at their face value, Hubble's constant probably lies within 12 units of 76 km s⁻¹ per megaparsec, but if the Local Group is really moving towards the Virgo cluster, Hubble's constant is probably more like 100 km s⁻¹ per megaparsec. This conclusion cannot easily be disputed; Hanes's own estimated errors are, with the benefit of recent observations, narrower than his predecessors have felt able to quote. One snag, of course, is that a large Hubble's constant means a younger visible universe, which raises difficulties for those concerned with the time needed for stellar evolution. But the conflict is not sharp enough to keep people awake at nights.

So what happens next? The ideal is that somebody should find a way of correlating some property of a distant galaxy with its intrinsic luminosity that will provide a distance scale as reliable as that of the Cepheid variables within the local galaxy; that would be sheer luck. Otherwise, further refinement will depend on more painstaking statistical analysis of that which Hanes describes. This is why Hanes's own account of his proof that Sandage and Tammann were mistaken is curiously sour — it is replete with words and phrases such as “wrong”, “incorrect”, “untested assumption”, “gives no assurances of the correctness” and so on. Hanes has every right to be proud of his latest paper on the extragalactic distance scale, for it is a thorough piece of work. But is he right to be so scornful of those whose even more careful work made possible his provocative reanalysis? Even Newton, after all, acknowledged that he had stood on the shoulders of giants.

Trouble in the J-land

from Jan Klein and Zoltan A. Nagy

IMMUNOLOGY is not exactly noted for its lack of controversies, but the publication of the paper by Steinmetz and his colleagues¹ in this issue of *Nature* (p.35) gives the controversies a new dimension. The authors have cloned a piece of mouse chromosome containing the class II genes of the major histocompatibility complex (MHC) and report, among other things, that they cannot find at least two genes placed into this piece by immunogeneticists. Now, not finding a gene would not cause much of a stir — immunologists have been quite frivolous about putting genes into the MHC and then taking them out again — were it not for the fact that one of them is the so-called *I-J* gene (*J* for short).

The *I-J* gene was described by Murphy and his colleagues² in 1976. They produced an antiserum, [B10.T(6R) × B10.D2]F₁ anti-B10.AQR, which contained only antibodies specific for MHC class II antigens. Since at the time class II molecules (which provide a context for recognition of antigens by T cells; see *News and Views* 297, 628) were believed to be present on B but not T lymphocytes, this antiserum was not expected to react with any T cells. However, when Murphy and his colleagues added it, together with complement, to a cell suspension containing suppressor T cells (cells capable of inhibiting the immune response of other cells), they discovered that the suppressive activity of these cells was abolished. When they then absorbed the antiserum by B10.A(5R) and B10.A(3R) cells, they found, to their surprise, that the former strain did and the latter did not remove the anti-suppressor T cell activity. The result was surprising because the B10.A(3R) and B10.A(5R) strains had been thought to be identical in their MHC: both were derived by intra-MHC crossing-over from the same two parental strains, C57BL/10 and B10.A, and the recombinations were believed to have taken place in approximately the same region. If we designate, as was then the custom, the MHC genes of the two parents as $K^b I-A^b I-B^b I-C^b S^b D^b$ (C57BL/10) and $K^k I-A^k I-B^k I-C^k S^k D^k$ (B10.A), then we can write the genotypes of the two recombinants as $K^b I-A^b I-B^b I-C^d S^d D^d$. The absorption data, however, indicated that the two recombinants were not identical and that the B10.A(5R) recombinant inherited from the B10.A parent something that the B10.A(3R) strain did not (the genotype of the B10.AQR strain, against which the antiserum was produced, is $K^q I-A^k I-B^k I-C^d S^d D^d$), and that this 'something' was controlled by a gene located between the *I-B* and *I-C* subregions. The authors designated this new subregion by the next available letter, *I-J*.

At the same time, another group of

investigators — Tada and his colleagues³ — reached a similar conclusion using a slightly different assay for the purported *J*-specific antibodies. They reported earlier that the inhibitory activity of the suppressor T cells on other cells was mediated by a soluble factor. When they incubated the supernatant containing the factor with an antiserum similar to that used by Murphy and his colleagues, the supernatant no longer inhibited the response, presumably because the suppressor factor was removed by the *J*-specific antibodies.

These discoveries were quickly confirmed by other laboratories and the *J*-specific antibodies became firmly entrenched in the mouse MHC map and in immunologists' minds. Eventually at least one commercial company began selling anti-*J* sera and at least two reports appeared in the literature describing the production of *J*-specific monoclonal antibodies^{4,5}. The original observations were then extended and the *J* determinants demonstrated on suppressor T cells and factors in a variety of systems. It appeared that not all suppressor T cells carried the *J* determinants and, in fact, the determinants were soon accepted as markers for a subpopulation of suppressor T cells. On the other hand, the determinants were also found on certain helper T cells and on macrophages. Furthermore, the *J* determinants were reported to stimulate mixed lymphocyte reaction, to restrict the interaction of suppressor and helper T cells, to be involved in the potentiation of immunity against syngeneic tumours, to govern the induction of immunological tolerance, as well as to do other remarkable things (see ref.6 for review). We have not counted the studies in which *J*-specific reagents have been used but there must be several hundred of them. In this situation, to question the orthodoxy of the *J* locus is likely to be regarded by many immunologists as a heresy of the most dangerous kind.

Yet, from the beginning, one could have had reservations about the *J* locus. For example, although *J*-specific antibodies had a potent effect in biological tests, they were extremely difficult to work with in serological assays. Some laboratories reported success in detecting *J* determinants by serological methods⁷⁻⁹ but in most immunologists' hands, the cells binding the *J*-specific antibodies could not be visualized. The explanation offered was that the *J* determinants were only on a small subpopulation of lymphocytes and

that the reactivity with these cells was obscured by the background reactivity of the serological assays. However, suppressor T cells can be enriched and purified and *J* determinants have been reported to be expressed by permanent cell lines and clones^{10,11}, so on these cells there should not have been any difficulty in detecting these determinants serologically. Even more disturbing was the fact that no one had succeeded in isolating the *J* molecules from the cell membrane. Here again, the availability of lines purportedly expressing *J* molecules should have provided suitable material for this isolation. Finally, there was the difficulty of the interpretation of the *J* locus. The designation *I-J* implied that *J* was a class II locus but, as we have pointed out on another occasion¹², there was no good reason for this classification. At best, the *J* determinants could be regarded as markers for a subpopulation of certain immune cells, but that they functioned in the same way as class II determinants do has never been satisfactorily demonstrated.

The situation with the other missing gene — the *B* gene — is far more clearcut than that with the *J* gene. The *B* gene was invoked to explain a particular pattern of responsiveness to a small number of antigens, a pattern involving the strains already mentioned, as well as another intra-MHC recombinant, B10.A(2R)¹³. No other trait could be associated with the *I-B* subregion and no *B*-specific antibodies could be produced by cross-immunization of strains purportedly differing at the *B* locus. Only a few immunologists felt compelled to invoke the *B* gene and when they did, it was only because they did not bother to think of a better explanation. That an alternative explanation did exist and that it was far more attractive than the old one was demonstrated by Baxevanis and his colleagues¹⁴. These investigators showed that the same pattern of responsiveness can be obtained without the postulate of the *B* gene but with the assumption that suppressor cells are involved in the control of the response. They then went on to accumulate convincing evidence that the alternative explanation was indeed the correct one.

Steinmetz and his colleagues have now laid the lingering apprehension about the *J* and *B* loci to rest. They have isolated the DNA from the B10.A(3R) and B10.A(5R) strains, the two strains that were used to define the *J* locus genetically, and have been able to ascertain from which parent the DNA segments around the purported *I-J* subregion have come. As expected they have found that the left-hand (centromeric) segment of these recombinants is derived from the B10 parent and the right-hand (telomeric) segment from the B10.A parent. Between these two segments they have found a region whose origin they cannot determine, and it is this region that should contain not only the *J* but also the *B* locus (in the rest of

Jan Klein and Zoltan A. Nagy are in the Max-Planck-Institut für Biologie Abteilung Immunogenetik, Corrensstrasse 42, 7400 Tübingen, FRG.

the DNA, the B10.A(3R) and B10.A(5R) are identical). The trouble is that this middle region is only 3.4 kilobases long and at least part of this length constitutes another gene, the well defined E_β gene. When one subtracts the minimal sequence that apparently is E_β , all that is left for the J and B genes is a sequence of less than 1,000 nucleotides. Such a sequence is, of course, far less than is required for any full-fledged gene, not to mention two such genes. It may, in fact, turn out that the B10.A(3R) and B10.A(5R) strains have identical or nearly identical DNA sequences around the site where the crossing-over occurred. In plain language, it may turn out that the J and B genes do not exist in the place where they were supposed to be. What the Steinmetz study already clearly establishes is that if the J and B genes do exist in this region, they must be quite unusual genes.

Steinmetz and his colleagues offer some graceful and some not so graceful ways out of this dilemma. The one possibility that they do not consider is that the B and J

genes simply do not exist. For the B genes, this possibility is not difficult to accept: after the demonstration by Baxevanis and his colleagues¹⁴ that the B locus is an illusion generated by a particular type of interaction between the suppressor and helper T cells, there has really not been any justification for keeping this locus in the MHC map. But the J gene? Is it possible that here, too, immunologists have fallen victims to some other kind of illusion? □

1. Steinmetz, M. *et al.* *Nature* **300**, 35 (1982).
2. Murphy, D.B. *et al.* *J. exp. Med.* **144**, 699 (1979).
3. Tada, T., Taniguchi, M. & David, C.S. *J. exp. Med.* **144**, 713 (1979).
4. Kanno *et al.* *J. exp. Med.* **154**, 1290 (1981).
5. Waltenbaugh, C. *J. exp. Med.* **154**, 1570 (1981).
6. Murphy, D.B. *Springer Sem. Immunopathol.* **1**, 111 (1978).
7. Parish, C.R. & McKenzie, I.F.C. *J. exp. Med.* **146**, 332 (1977).
8. Okumura, K. *et al.* *J. exp. Med.* **146**, 1234 (1977).
9. Hayes, C.E. & Bach, F.H. *J. exp. Med.* **148**, 692 (1978).
10. Taniguchi, M., Saito, T. & Tada, T. *Nature* **278**, 555 (1979).
11. Okuda, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **78**, 4557 (1981).
12. Klein, J. *et al.* *Nature* **291**, 5815 (1981).
13. Leiberman, R. *et al.* *J. exp. Med.* **136**, 1231 (1972).
14. Baxevanis, C.N., Nagy, Z.A. & Klein, J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3809 (1981).

Mapping the Universe

from Virginia Trimble

OUR inventory of basic kinds of objects and structures in the Universe is probably not yet complete, even on the largest scales. Thus participants in IAU Symposium 104 on 'Early Evolution of the Universe and its Present Structure'* heard of a new class of radio sources, of changes in our best estimates of irregularities in the 3K microwave background radiation, and much debate on the morphology and origin of superclusters of galaxies and the relative voids between them. The other main topic of discussion, exotic physics in the very early universe, was recently reviewed in these pages by Barrow and Turner¹.

For a quarter of a century the counting of radio sources has been telling us both about the nature of the sources and the large-scale structure of the Universe². Essentially, as we count from the brightest sources towards fainter ones, we at first see more sources than expected if the objects are distributed uniformly in euclidean space, and then later we see less sources than would be predicted. J. Peacock (Royal Observatory, Edinburgh) reiterated the standard interpretation — powerful radio sources in elliptical galaxies and quasars were commoner in the past than now, but that very few turned on before the time corresponding to a redshift of about 4. P. Osmer (CTIO, Chile), A. Braccisi (University of Bologna) and A. Savage (UK Schmidt Telescope Unit) reported optical searches for quasistellar objects reinforcing this conclusion, though the last

of the three groups involved has just reported a new record quasar redshift³, $z = 3.78$ for PKS2000-330.

But, as we look to still fainter radio sources, the downward trend of number versus apparent brightness ceases, according to a new series of source counts, carried out at 1,412 MHz by R. Windhorst and others at Westerbork, and reported by H. van der Laan (University of Leiden). The most likely interpretation of this flattening in number versus flux is a new class of radio sources, which van der Laan called 'Population II radio galaxies'. They make up some 75 per cent of the sources seen at 1,412 MHz fluxes near 1 mJy, and their optical identifications are typically faint, fuzzy blue galaxies of unknown type. The radio morphology is both less tidily structured and smaller (≤ 50 kpc) than that of brighter sources. The clustering properties are unknown. Van der Laan said that the new sources might be either spiral galaxies at redshifts near 0.4 or (at long last) the radio emission from so-called radio-quiet quasars, since the luminosity function is much like that of standard radio sources, except it is fainter by a factor of 1,000. The IR colours, according to Windhorst, divide into two classes, resembling those of giant elliptical radio galaxies and of less luminous spiral galaxies respectively. E. Fomalont and J. Wall presented results of an NRAO survey at 6 cm that also shows some flattening of number versus flux at the lowest flux levels surveyed and thus provides independent evidence of this (or at least some) new

population of sources.

Looking for structure in the 3K background radiation is necessarily a less ancient activity than counting radio sources, though it is coeval with the discovery of the radiation itself⁴. N. Parijskij (Special Astrophysical Observatory), F. Melchiorri (Rome), D. Wilkinson (Princeton University), K. Kellermann (NRAO) and A. Lazenby (Jodrell Bank) reported on searches for anisotropies on various size scales and at various wavelengths carried out in the USSR, Italy, the US and Great Britain. The main conclusions follow.

First, the dipole anisotropy, attributable to our motion relative to the particles that last scattered the radiation, is here to stay. Though there is still some discrepancy between the Berkeley and Princeton results (owing, apparently, to calibration errors), we can say that the Sun is moving at 372 ± 25 km s⁻¹ towards right ascension 11.2 h, $\delta = -6^\circ$. This translates into a ~ 600 km s⁻¹ velocity for the Local Group of galaxies, aimed about 30° away from the Virgo cluster, and so is at least roughly consistent with the result reported by J. Mould (Kitt Peak) from optical observations of galaxies of a few hundred km s⁻¹ infall towards Virgo plus ~ 180 km s⁻¹ rotation around it. The dipole anisotropy shows a small variation with a period of one year. Copernicus was right!

Second, the quadrupole anisotropy reported by two groups^{5,6} has gone away and was apparently an artefact of improper subtraction of the contribution from continuum radiation by our own Galaxy. It has left behind a bright spot outside the galactic plane in the 0.6-2 mm data of the Rome group⁷ but not in the longer-wavelength data from Berkeley and Princeton. Wilkinson suggested the hot spot might be thermal radiation by a large galactic dust cloud, though Melchiorri suspects it may have cosmological significance.

Third, on smaller scales, there continue to be mostly upper limits, $\Delta T/T \leq 3 \times 10^{-4}$ for $1'$ to 1° scales from the NRAO and Jodrell surveys and $\leq 3-5 \times 10^{-5}$ on similar scales from the Rattan 600 (USSR) survey. G. de Zotti (University of Padova) suggested that the second of these limits could not be correct, because known radio sources alone should contribute larger fluctuations; and the American and British observers expressed audible curiosity about how the measurements were made. Parijskij's response was that the Rattan telescope is simply more suitable than most others for work on extended emission. Even the higher limits are already serious constraints on our models for cluster/galaxy formation because they pertain to the angular size of clusters at the

Virginia Trimble is Visiting Professor of Astronomy at the University of Maryland College Park, Maryland 20742 and Professor of Physics at the University of California, Irvine, California 92717.

*The symposium was held 30 August-2 September in Chania, Crete.

largest redshifts at which adiabatic perturbations in the matter density can begin to grow — we must have reasonable lumpiness then to get the lumpiness we see now. Ways out of this dilemma include, according to E. Salpeter (Cornell University), B. Jones (University of Paris), S. Bonometto (University of Padova) and N. Kaiser (University of Cambridge), an initial spectrum of fluctuations whose shape was not a power law; re-ionization at $z > 10$ to smooth out the lumps in the radiation but not the matter; galaxy and cluster formation by some process that makes the necessary lumps after recombination, as suggested by Ostriker and Cowie⁸; massive neutrinos, which can begin to clump as much as they want without distorting the radiation once they stop interacting with the photons (which happens much sooner for them than for ordinary matter); or non-adiabatic perturbations (which are, however, not permitted by modern grand unified theories¹).

On all scales, we cannot hope to do much better than present accuracies and limits, which, according to Wilkinson, are within a factor of 3–4 of noise levels.

The study of superclusters (clusters of clusters of galaxies) and the voids between them is the oldest of all these activities; for, as J. Oort (University of Leiden) pointed out, their existence and main properties are already implicit in the Shapley–Ames catalogue⁹ of 1932. There is general agreement on the existence of structures of up to 100–150 Mpc, separated by about twice that amount. The structures are at least somewhat elongated, with crossing times on the long axis greater than or approximating the age of the Universe and 10^{15} – 10^{17} the mass of the Sun. And the density contrast between supercluster and void is sufficient (at least factors of 2–4) that we see some deceleration of the general Hubble expansion of the Universe at the edges. The chief observational items of dispute are (1) just how empty are the voids, (2) whether the typical supercluster shape is an ellipsoid, a pancake, or a string, and (3) the extent to which the superclusters join up into a network or cell structure. These bear directly on the chief theoretical question of how the superclusters formed. The competing models present explanations in terms of gravitational clustering of smaller-scale structures or fragmentation within pancake-like gas sheets formed by nonlinear growth of adiabatic perturbations.

Both models can make superclusters with roughly the correct size and correct first- and second-order correlation functions¹⁰. But the pancake models should result in emptier voids, more stringy features and more networking than the clustering models^{14,15}.

Looking into the voids, S. Shethman (Mt Wilson) reported an extension of the Kirshner *et al.*¹¹ redshift survey that

confirms the existence of a sphere 120 Mpc across (for $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$) centred at right ascension 15 h, $\delta = +50^\circ$, $V_r = 15,000 \text{ km s}^{-1}$ in which the density of galaxies is at most one quarter that of the surroundings. In fact, they find no galaxies at all in that sphere, though there are apparently modest numbers of emission line galaxies^{12,13} in adjacent zones previously thought to be empty.

As far as shapes of superclusters go, our own Virgo one is a 6:3:1 ellipsoid according to R. Tully (Hawaii). But it is apparently far from typical. J. Einasto (Tartu) and F. Shandarin (Institute of Applied Mathematics, Moscow) reported applications of a new (to most astronomers!) technique, called percolation theory¹⁶, which is particularly suited to determining topology (degree of connectedness) of structures, rather than just the amount of clustering. The primary conclusions are that the basic structures are strings of clusters and galaxies with a filling factor of about 1 per cent; that essentially all the strings are connected into a network; that the luminosity contrast between strings and voids is at least 10^3 , though there are small strings within voids like the one of Kirshner *et al.*¹¹; and that no one model copes simultaneously with all the data.

Finally, R.H. Miller (ESO and Univer-

sity of Chicago) showed cinematically the results of assorted numerical clustering experiments, some of which seemed to the eye to have produced much the same sorts of filaments and cells as seen in the distribution of real galaxies, though this has not yet been tested by percolation theory or anything similar. But he left us with the important thought that, just because simulation of a process produces something that looks like the real world, this does not necessarily mean that nature did it that way! □

1. Barrow, J.D. & Turner, M.S. *Nature* **298**, 801 (1982).
2. Ryle, M. *IAU Symp.* **4**, 221 (1957).
3. Peterson, B.A., Savage, A., Jauncey, D. & Wright, A. *Astrophys. J. Lett.* **260**, L27 (1982).
4. Penzias, A. & Wilson, R.W. *Astrophys. J.* **142**, 418 (1965).
5. Fabbri, R., Guido, I., Melchiorri, F. & Natali, V. *Phys. Rev. Lett.* **44**, 23 (1980).
6. Bough, S., Cheng, E.S. & Wilkinson, D.T. *Astrophys. J. Lett.* **243**, L113 (1981).
7. Melchiorri, F. *et al. Astrophys. J.* (in the press).
8. Ostriker, J.P. & Cowie, L.L. *Astrophys. J. Lett.* **243**, L127 (1981).
9. Shapley, H. & Ames, A. *Harvard Ann.* **88**, Pt II (1932).
10. Aarseth, S.J. & Saslaw, W.C. *Astrophys. J. Lett.* **258**, L7 (1982).
11. Kirshner, R.P., Oemler, A., Schechter, P.L. & Shethman, S. *Astrophys. J. Lett.* **248**, L57 (1981).
12. Balzano, V.A. & Weedman, D.W. *Astrophys. J. Lett.* **255**, L1 (1982).
13. Sanduleak, N. & Pesch, P. *Astrophys. J. Lett.* **258**, L1 (1982).
14. Doroshkevich, A.G., Shandarin, S.F. & Zel'dovich, Ya. B. *Commun. Astrophys.* **9**, 265 (1982).
15. Arnold, V., Shandarin, S.F. & Zel'dovich, Ya. B. *Geophys. Astrophys. Fluid Dyn.* **20**, 111 (1982).
16. See, for example, Shante, V.K. & Kirkpatrick, S. *Adv. Phys.* **20**, 325 (1971).

Air pollutants act in combination

from J.N.B. Bell

NEW work from the University of Lancaster reported in this issue of *Nature* (p.55) highlights the importance of the combined effects of NO_2 and SO_2 — Britain's two most widespread air pollutants — in reducing the growth of grasses and trees. The work demonstrates the complexity of growth responses to SO_2/NO_2 mixtures and the danger of attempting to predict injury in the field on the basis of the single harvest fumigation experiments which are invariably employed as the basis for developing air-quality criteria.

Whitmore and Freer-Smith¹ fumigated *Poa pratensis* (meadow grass) and cuttings or seedlings of six tree species, *Malus domestica* (apple), *Betula pendula* (silver birch), *Betula pubescens* (common birch), *Populus nigra* (black poplar), *Alnus incarna* (grey alder) and *Tilia cordata* (small-leaved lime), for up to eleven months in outdoor glass chambers, in the presence of SO_2 , NO_2 , and SO_2 and NO_2 at concentrations (0.062 p.p.m.) encountered

in some urban regions. Growth rates were compared with those of plants in clean air.

When *P. pratensis* was fumigated overwinter, the three gas treatments produced markedly different results between November and February — SO_2 had relatively little effect, NO_2 stimulated growth, and the mixture produced a substantial fall in dry weight. The greater than additive effect of the gas mixture subsequently disappeared, and reductions in growth became apparent in all treatments, although they recovered to the same level as the controls by June. A shorter summertime fumigation of the trees produced a greater than additive effect with SO_2 and NO_2 in four out of the six species.

That the air of suburban areas of British cities contains pollutants that can cause large reductions in the growth of grass species has been demonstrated by comparing the growth of plants grown in chambers ventilated with ambient or clean air^{2–4}. Such effects may occur when the mean SO_2 level is much lower (0.011–0.026 p.p.m.) than that which reduces plant growth in fumigation experiments (≥ 0.038 p.p.m.)⁵, suggesting that the fumigation design does not take into account the

J.N.B. Bell is in the Department of Pure and Applied Biology and the Centre for Environmental Technology, Imperial College of Science and Technology, London SW7 1LU.

presence of other pollutants and the continuous fluctuation of SO_2 in ambient air⁶.

Relatively recently, fumigation experiments have begun to be carried out using continuously fluctuating pollution levels. Two recent reports, however, indicate that the occurrence of peak concentrations of SO_2 may not be as important as has been suggested. Fumigations of *Phleum pratense* (timothy) with 0.06 p.p.m. SO_2 for 41 days produced the same effects on growth, irrespective of whether the pollutant was administered at a constant level, or with peak concentrations of 0.4 and 0.8 p.p.m. respectively⁷. Similarly, when *Pinus sylvestris* (Scots pine) seedlings were fumigated over 650 days with 0.038 p.p.m. SO_2 , given either at a constant level, or as four treatments comprising factorial combinations of high (0.281 p.p.m.) and low (0.113 p.p.m.) hourly peaks over short (5 h) and long (21 h) periods, the constant treatment resulted in a 14 per cent reduction in dry weight increment, and peak concentrations did not produce any further growth reduction, unless they were of 21 hour duration⁸.

It thus seems likely that the presence of other pollutants, in addition to SO_2 , is a major factor in reducing plant growth in cities. High-temperature combustion processes produce abundant quantities of nitrogen oxides (NO_x), which have hitherto been little studied, except for their role in the formation of photochemical oxidant smogs. NO_x is emitted into the atmosphere mainly in the form of NO, irrespective of whether the source is motor-vehicle exhaust or coal/oil-fired power stations, but is rapidly oxidized to NO_2 . The limited data available show that mean NO_x levels are equal or greater (v/v) than mean SO_2 concentrations in both urban and rural areas. A SO_2/NO_x ratio of 1:1.5 has been recorded for central London⁹ and it has been suggested¹⁰ that approximately 11 per cent of rural land in western Europe may be exposed to SO_2 and NO_2 concentrations of > 0.038 and > 0.042 p.p.m. respectively. Clearly there is a need for the incorporation

of NO_2 (and possibly NO) into fumigation experiments designed to investigate SO_2 effects on plants. Both additive and more than additive responses have been observed for SO_2/NO_2 mixtures on different grass species¹¹ but the work of Whitmore and Freer-Smith demonstrates for the first time that such effects are markedly influenced by season.

Considerable difficulty has been experienced in determining thresholds for long-term SO_2 injury to grass species¹². Interactions between changing environmental conditions and SO_2 effects have

been suggested as the main factor obscuring a clear dose-response relationship. Whitmore and Freer-Smith have identified another important factor, by showing that SO_2 , NO_2 and their mixture have very different effects if the fumigation is terminated at different times of the year. Their work demonstrates the need for further such long-term experiments, with harvesting at intervals and, if possible, extension over several growing seasons in order to determine the net result of the positive and negative growth effects of the pollutants. □

Transformation and gene organization in *Drosophila*

from Michael Ashburner

THE highlight of Crete III, the third EMBO-sponsored workshop* on the molecular biology of *Drosophila*, was, without doubt, the announcement by G. Rubin and A. Spradling (Carnegie Institution, Baltimore) that they had successfully transformed *Drosophila* with exogenous DNA — results that have just made their appearance in *Science* (October 22, p.348). Moreover, D. Ish-Horowicz (Imperial Cancer Research Fund, London) reported that, in collaboration with J. Sang's group (University of Sussex), the transformation of *Drosophila* tissue culture cells with DNA had also been achieved.

The flies were transformed by the injection of exogenous DNA into embryos using the transposable P element as a vector. The P elements of *Drosophila* are repetitive genetic elements found only in certain strains (so-called 'P' strains) and absent from other ('M') strains. In hybrids between a P strain and an M strain, the P elements contributed by the P parent may become highly unstable (especially if the cross was between a P father and an M mother), to produce the syndrome of hybrid dysgenesis (see *News and Views* 299, 676; 1982).

Rubin and Spradling constructed a vector that contained a wild-type *rosy* gene sequence within a P element sequence. Flies mutant for *rosy* have brownish eyes, since they lack the xanthine dehydrogenase (XDH) needed to synthesize the normal red eye pigments. *rosy* is particularly useful for this sort of experiment as only a few per cent of normal XDH activity is necessary to ensure a wild-type eye colour and the gene need not be active in the 'correct' tissue (or, perhaps, even at the 'right' stage of development) for a wild-type eye colour to

result. Two successful methods of transformation were described. In the first, *rosy*-P plasmid was injected into embryos that were both mutant for *rosy* and carried their own integrated P elements on a dysgenic background. In the other, the *rosy*-P plasmid was co-injected with a cloned intact P element into non-dysgenic, and non-P-carrying, embryos. The introduced *rosy* genes integrated into the genomes of their hosts and at least one was active enough to ensure a wild-type eye colour.

The only other genes which have, so far, been used to transform flies are those coding for the egg-shell proteins. These are of particular interest for they amplify during normal development in the ovarian follicle, the tissue that secretes the egg shell. Spradling discussed the results of transformation with two different egg-shell protein gene fragments. In the transformed fly strains, three to four copies were integrated at various chromosome sites. One of these fragments amplifies according to its normal developmental schedule, despite its unusual location in the genome. This promises to be a powerful method for the study of those sequences responsible for the normal tissue-specific amplification of these genes.

Transformation was not, of course, the sole topic of discussion in Crete. Studies on the molecular organization of genes in *Drosophila* now seem to indicate that there are several different discrete categories of gene. The simplest are those present in a single copy. Only two have been reasonably well characterized — *rosy* (coding for XDH; W. Bender, Harvard Medical School) and the gene for alcohol dehydrogenase (T. Benyajati, Frederick

1. Whitmore, M.E. & Freer-Smith, P.H. *Nature* 300, 55 (1982).
2. Bleasdale, J.K.A. *Environ. Pollut.* 5, 275 (1973).
3. Crittenden, P.D. & Read, D.J. *New Phytol.* 80, 49 (1978).
4. Crittenden, P.D. & Read, D.J. *New Phytol.* 83, 645 (1979).
5. Bell, J.N.B. in *Effects of Gaseous Air Pollution in Agriculture and Horticulture* (eds Unsworth, M.H. & Ormrod D.P.) 225 (Butterworth, London, 1982).
6. Mansfield, T.A. & Freer-Smith, P.H. *Biol. Rev.* 56, 343 (1981).
7. Jones, T. & Mansfield, T.A. *Environ. Pollut.* A28, 199 (1982).
8. *Clean Air* 9, 61 (1979).
9. Garsed, S.G., Mueller, P.W. & Rutter, A.J. in *Effects of Gaseous Air Pollution in Agriculture and Horticulture* (eds Unsworth, M.H. & Ormrod D.P.) 455 (Butterworth, London, 1982).
10. Fowler, D. & Cape, J.N. in *Effects of Gaseous Air Pollution in Agriculture and Horticulture* (eds Unsworth, M.H. & Ormrod, D.P.) (Butterworth, London, 1982).
11. Ashenden, T.W. & Mansfield, T.A. *Nature*, 273, 143 (1978).
12. Bell, J.N.B., Rutter, A.J. & Relton, J. *New Phytol.* 83, 627 (1979).

*The 3rd Workshop on 'The Molecular Biology and Developmental Genetics of *Drosophila*' was held at Kolymbari, Crete, on 21–26 June 1982 and was sponsored by EMBO and the University of Crete.

Michael Ashburner is at the Department of Genetics, Downing Street, Cambridge CB2 3EH.

Cancer Institute, Maryland; M. Ashburner and colleagues, University of Cambridge).

Yet even these genes may not be that 'simple'. At *Adh*, for example (Benyajati; N. Spoerel, University of Cambridge), transcription occurs from two quite different promoters in different tissues and at different stages of development.

Several 'complex' genes — complex in terms of their genetic organization and their developmental phenotypes — have been identified by *Drosophila* geneticists. Some of them are now cloned: *bithorax* (Bender; M. Akam, University of Cambridge; R. Saint, Stanford University), *Notch* (M. Young, Rockefeller; S. Artavanis-Tsakonis, Yale University), *scute* (J. Modolell, University of Madrid) and *rudimentary* (K. Louis, Princeton University) are examples. All are very large (*scute* is spread over at least 60 kilobases) and all produce a bewildering variety of developmentally regulated transcripts — at least four have been found at *scute*, each with quite different developmental specificities.

A third class of gene may be called 'clustered multicopy'. The copy number is low, from two to less than ten, the copies are all contained within a relatively short stretch of DNA (few tens of kilobases) and the different copies have often diverged so that they code for different, but functionally related, proteins. Several examples are known: the four genes coding for the small heat-shock proteins (E. Craig, University of Wisconsin-Madison), three genes coding for salivary gland glue proteins (E. Meyerowitz, Caltech) and the two major chorion protein gene clusters (Spradling; F.C. Kafatos, Harvard University) are well worked out. Presumably the members of a cluster are related by descent. However, not only are they often transcribed from different strands of the DNA but the extent of divergence since their presumed duplication may be considerable. Of the three different glue genes, one codes for a protein of 284 residues and the other two for much smaller proteins (about 50 amino acids). The large protein consists of four domains — a hydrophobic leader, 44 amino acids very high in threonine, 37 copies of a five-residue repeat and a C-terminal domain of 50 residues, very high in cysteine. The small proteins 'simply' lack the internal two domains! (Meyerowitz).

A fourth class of gene is the 'dispersed multicopy'. Again copy number is low, less than ten, but the copies are dispersed around the genome. If there are rules governing their location we do not know them yet. Examples are the actin genes (N. Davidson, Caltech), the genes coding for the larval serum proteins (four genes; D. Roberts, University of Oxford) and the *Hsp68* and *Hsp70* heat-shock genes and their cognates (Craig). Again, studies of the different copies suggest that they have evolved by duplication. Presumably (but

not necessarily) they have been dispersed subsequently by a transposition process. Why some genes should be organized in small clusters and others dispersed is not yet clear. Of course some genes, for example, *Hsp 70*, share characteristics with both classes, being dispersed clusters. Finally, there is the class of tandemly repeated, high-copy number genes such as those coding for the rRNAs and the histones.

A fascinating family of transposable elements (TE) has been discovered by Bender. Called 'gypsy', they are often associated with spontaneous mutations that share the property that they can be suppressed by a recessive mutant allele of *su(Hw)*. Presumably gypsy acts at a distance, since revertants of some gypsy-associated *bithorax* mutations are only partial losses of the gypsy element itself. The suppressor does not act by causing the element to excise. Of quite a different order from these elements is G. Ising's (University of Lund) TE, which is vast (> 100 kb) and carries at least two genes (*white* and *roughest*). R. Paro (University of Basel) has cloned some examples of this TE: its transposable property apparently results from long 'fold-back' sequences at its ends. This 'FB-NOF' sequence also occurs elsewhere in the genome, for example the

unstable *white-crimson* mutation is associated with this element.

Although molecular biology dominated the meeting it must not be thought that the gene-jockeys had it all their own way. An elegant demonstration of the error of inferring too much from the phenotype of mutations in a complex eukaryote such as *Drosophila* came with B. Baker's (La Jolla) study of mitosis in a random selection of conditional lethal mutations. Many had been selected for some 'interesting' developmental phenotype, for example an absence of imaginal disks. A suprisingly high proportion of the mutations turn out, however, to disrupt mitosis and some are very dramatic — accumulating over 1,000 chromosomes per nucleus. All diploid cells are affected. Although the mutants were originally called 'diskless' because this is the most prominent feature of their phenotype, they are clearly not 'imaginal disks' mutations in anything but a rather trivial sense.

The vigour of modern *Drosophila* biology was evident at Crete. The discovery of new molecular and genetic techniques cannot but fail to keep *Drosophila* research in the forefront of modern biology. This, and the patronage of the Crete meetings by the Bishop of Kastelli Kissamou, augurs well for Crete IV. □

Food — the best medicine

from Ben J. Miflin

EVERY so often society rediscovers the obvious. Currently many sections of society from Wall Street to Westminster are rediscovering plants and man's dependence on them. The waves of enthusiasm emanating from the new converts have led to the founding of numerous companies in the US and may yet be strong enough to push the British Technology Group into launching Celltech's country cousin (*Nature* 297, 5; 1982). But where to sail? What to do? What to improve? Many think-tanks have pondered these questions and the CIBA Foundation* has recently broken its long-standing tradition of medically based symposia to address the topic of 'Better Crops for Food'.

First, define 'better', define 'food'! The answer depends on where you are standing and on how carefully you look at a particular agricultural system (C. Spedding, University of Reading). An apparent improvement may work in one context, be irrelevant in another and be absolutely disastrous in a third. Only if there is some appreciation of how the different systems work is there a chance of

predicting, usually on the basis of modelling, what the effect of a development might be or which changes might be desirable.

Man has catalogued only a proportion of the world's plant species and he depends on 15 or so for the bulk of his agriculture (E. Bell, Royal Botanic Gardens, Kew). Many plant species are being lost irretrievably and these include reservoirs of germplasm relevant to our crops. However, what and how we should conserve is a matter of controversy. One answer may be the application of cryogenic techniques for storing plant tissue cultures (K. Dougall, University of Tennessee), but predicting what may be useful in the future is uncertain. New crops may emerge but the conservatism of agricultural and cultural practices limits likely crops to those which are already grown to some extent in marginal areas. The winged bean (*Psophocarpus tetragonolobus*) is one likely candidate; it seems to have many virtues including protein-rich tap roots and metre-long pods that can be eaten green or dried (N. Haq, University of South-

* The Ciba Foundation meeting on 'Better Crops for Food' was held 14–16 September. The proceedings will be published as Ciba Symposium 97 (Pitman Books).

Ben J. Miflin is in the Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ.

ampton; S. Karikari, Ahmadu Bello University, Nigeria). Traditional intercropping systems practised in developing countries may also be a more appropriate guide to future cropping systems than western monoculture, as studies at ICRISAT have shown (R. Willey, University of Reading). The advantages may not be restricted solely to the tropics as mixed cropping of cultivars of barley and wheat, developed to combat disease, may yield more even in the absence of disease (P. Day, Plant Breeding Institute, Cambridge, quoting results of Wolfe).

The aims in breeding better crops are usually maximum yield (although maximum financial return may be better), disease resistance and nutritional quality. Improved yield has generally resulted from improved harvest index rather than from changes in carbon dioxide fixation rates. Nutritional quality in legume seed depends on many components (D. Boulter, University of Durham) and genotypes of soybeans lacking some of the undesirable ones have been found (T. Hymowitz, University of Illinois). Whether it is worth incorporating these traits into commercial cultivars depends on the rest of the diet, how the beans are cooked and whether the components confer pest or disease resistance on the seed.

Increases in yield, particularly of cereals, has resulted equally from improved cultivars and improved agronomic practice. Classical agronomic research started in the UK at Rothamsted with the aim of improving soils to fit the needs of the crops available. The approach may be curtailed in the future by the lack of energy to produce fertilizers (E. Bell), although others (R. Hardy, Du Pont, Wilmington; B. Mifflin, Rothamsted Experimental Station) argued that this was unlikely as these inputs are a minor part of fuel use and often produce a positive energy return. Replacement of nitrogen fertilizer for cereals, by introduction into them of nitrogen fixation genes or by the use of associated symbioses, is unlikely other than in the very long term. However, rhizosphere microorganisms, including *Azospirillum*, may have an important role in enhancing nutrient uptake by crops (R. Hardy). Crops can also be tailored for the soils we have, whether they are naturally high in aluminium as in Brazil or made saline by continual irrigation as in California (E. Epstein, University of California, Davis). Energy to process crops into food may be the major input in both developed and developing countries; in the former it is often unnecessarily excessive, in the latter it can be a matter of life or death as when cooking inactivates toxins. Developing countries are also suffering an energy crisis due to the destruction of trees which, for many reasons, are important components of agricultural systems (P. Nair, International Council for Research in Agroforestry, Cambridge).

Thus there is no lack of problems. But

what of new tools to tackle them? The microchip makes computing on the farm possible and many systems are being developed to maximize efficient use of inputs; one such — EPIPRE — was described by J. Zadoks (Wageningen Agricultural University, The Netherlands). This predicts the optimum amount and timing of the use of crop-protection chemicals on a field-by-field basis. The result is mostly to reduce the amounts applied, thus reducing cost and preventing pollution — still further reductions will come with improvements such as electrostatic spraying. Practitioners of genetic engineering techniques, despite the current enthusiasm, generally do not see the dramatic production of 'super-plants' in the near future. What these techniques will do is improve our understanding of plant genetics and provide technical short-cuts for breeders. Flavell (Plant Breeding Institute, Cambridge) described two such applications for recombinant DNA techniques: for rapid measurement of genetic diversity in order to recognize desirable types and for a quick and sensitive assay to detect plant viruses. Each technique could shorten the long time required at present to breed new varieties. 'Intermediate' technologies which may be of short-term significance are those which produce genetic diversity, such as 'somaclonal' variation. This term, coined by W.

Scowcroft (CSIRO, Canberra) describes the variation released during de- and re-differentiation of plants through protoplast and tissue culture techniques which is currently being assessed for commercial promise in several crops. Further down the line are protoplast techniques, including somatic cell fusion, which have the promise of transfer of desirable traits across the barriers of sexual incompatibilities (O. Schieder, Max Planck Institute für Züchtungsforschung, Cologne).

By way of conclusion, Spedding's quotation from Poul Andersson seems appropriate "I have never encountered any problem however complicated which, when looked at in the proper way, did not become still more complicated". In producing more food to meet the demand of the world's rapidly rising population there is no one problem, no one answer, only an almost infinite range of possibilities. In these circumstances research priorities should be diverse enough to provide various alternative solutions to the most likely crises. Evidence that this is the route to success comes from plants themselves, as E. Bingham (University of Wisconsin-Madison) elegantly showed. Lucerne cultivars bred for maximum heterozygosity have maximum vigour; dependence on a narrow (genetic) base leads to weakness and depression. □

100 years ago

A Curious Halo

THERE appeared in *Nature*, vol. xxvi. pp.268, 293, two articles headed "A Curious Halo," which reminded me of an analogous and still more curious phenomenon occurring sometimes here in China, during the hot season. I beg to hand you a few lines on that subject, from the *Monthly Bulletin* of the Zi-ka-wei Observatory for August, 1877.

"A phenomenon to which I wish to call the attention of meteorologists was observed many times during that month (August), as also in July. It does not seem to take place in Europe, and I am inclined to think that it cannot occur except with an atmosphere over-charged with aqueous vapour, as it is the case with us in July and August. In the evening, just after sunset, or in the morning even long before sunrise, no matter what the direction of the wind and the barometric pressure may be, provided the day or night were very warm, bands of a tint varying from the faintest to the deepest blue are seen to appear upon the whitish or roseate vault of heaven. They usually are first seen in the east at evening and in the west at morning time, seemingly radiating from a common centre diametrically opposite the sun's position. At other times they emerge from the very position of the sun, or from both points at once, the interval being either free from bands or completely encircled by them.

"Last year (1876), on the morning of September 4, I enjoyed a most interesting sight. It was about 5 a.m., the moon, then on her nineteenth day, was above the western horizon, and just being partially eclipsed; now from her bright disc, as from a radiating centre, shot out a number of those bands or blue beams; they traversed the whole expanse of the sky, and seemed to converge towards a point whose situation in the east below the horizon corresponded with that of the moon in the west above the horizon.

"The bands or shoots are more or less numerous, bright, and persistent; some have been observed in the evening, fortyfive minutes after sunset, and in September, 1876, I saw them appear with the first break of day. They are evidently movable in the sky, and there is no doubt that they are due to comuli floating about the horizon, below or above, through which the light of the sun is sifted and split; they are, in fact, nothing else than the twilight. According as the clouds before the sun are more or less compact or loose, the bands may be blue, white, or red. More than once also have I seen the sky half white and half blue, the separation of the two colours being plainly perceivable, and Venus shining brilliantly in the blue sky close to that limit, whilst it would probably have been almost invisible through the milky sky hard by."

Any one who gazes for the first time at this beautiful phenomenon cannot help wondering and acknowledging it to be greatly different from anything to be seen elsewhere. It is quite certain that the phenomenon is due to the atmospheric vapour, but I am rather at a loss to give a more satisfactory explanation. The dispersion of the direct rays of the sun into the minute drops resulting from a partial but wide-spreading condensation of the aqueous vapour in the upper strata of the air, might account for evening time. Besides, the interposition of a light cloud in the way of the sun's rays does not impair the transparency of the drops, and the blue sky may be visible. Now, in the morning and evening the rays of the sun are almost parallel with the horizon; they traverse the whole expanse of the sky, and their apparent convergence on the both sides is only due to the same optical illusion which shows us the two rails of a railway track or the walls of a tunnel as converging.

MARC DECHEVRENS

Zi-ka-Wei Observatory, near Shanghai,
From *Nature* 27, 30, 9 November 1882.

Fire deaths and toxic gases

from Barbara Chernov Levin

AMONG the industrialized countries which record statistics on fires, the United States has the most fires per capita. The rate of fire death in the United States is almost twice the international average and is surpassed only by Canada¹. In 1979, approximately 7,800 people died and another 31,000 were reported injured². Direct dollar loss was greater than \$5 billion, but when indirect costs (meeting fire codes, installing fire protection systems, supporting fire departments and insuring property) are included in this calculation, the annual cost of fires in the United States is estimated at more than \$20 billion.

Large fires receive the most public attention and media coverage; for example, three hotel fires between November 1980 and May 1981 were the cause of 119 fire deaths and more than 1,000 injuries. Residential fires, however, continue to be the cause of most fire deaths and injuries. Approximately 80 per cent of these fire deaths are not due to burns, but are attributed to inhalation of toxic smoke and hot gases³. This information has prompted the fire community, materials manufacturers and building code officials to pose the following questions: do some building materials and furnishings produce combustion products that are much more toxic than others, and how can the occupants of buildings be protected from unnecessary risk in case of a fire? The answers to these questions are not simple and involve the evaluation of the total toxic hazard generated by a fire.

The potential for generating toxic gas from burning materials has long been recognized; for example, in the 1929 Cleveland Clinic fire, 123 people were killed by the decomposition products of nitrocellulose X-ray film⁴. Only recently, however, has the fire research community become acutely aware and concerned about the problem of the toxicity of combustion products and the possibility that toxic gases other than carbon monoxide (still a primary toxicant in fires) may have an important role. Autopsy data show a sample of fire victims with less than lethal amounts of carbon monoxide in their blood^{5,6}. Other factors may have been involved, however, as some of the victims had pre-existing heart disease and/or alcoholic intoxication.

Laboratory research studies also suggest a role for toxicants other than carbon monoxide. A highly toxic bicyclic phosphate compound produced during the thermal decomposition of a laboratory-formulated rigid flame-retarded polyurethane foam was identified by the University

of Utah⁷ and suggests the need for an animal testing system — conventional chemical techniques would have failed to detect such an unusual toxic product. Moreover, scientists at the National Bureau of Standards have found that carbon monoxide concentrations measured at the LC₅₀ of twelve thermally decomposed materials were 60–4,400 p.p.m., whereas the LC₅₀ of pure carbon monoxide was 5,000 p.p.m. The LC₅₀ here measures the amount of material necessary to kill 50 per cent of the rats exposed for 30 min. The death of the rats at less than the LC₅₀ of pure carbon monoxide indicated that additional toxic gases or other factors (for example, heat, lowered oxygen concentrations) were contributing to the cause of death. Many of the exposed rats also died during a 14 day post-exposure observation period, an occurrence which cannot be directly attributed to carbon monoxide poisoning.

Some of these thermally decomposed materials also produced the potent toxicants hydrogen cyanide and/or hydrogen chloride as well as carbon monoxide. Chemical analysis has detected many combustion products in addition to the frequently examined toxicants — carbon monoxide, carbon dioxide, hydrogen chloride, other halogens, oxides of nitrogen and sulphur, and ammonia. Scientists at the Fire Research Station in England have identified 20 chemical species from the thermal decomposition of polyacrylonitrile and 30 combustion products from polypropylene⁵. Michal *et al.* have identified 71 compounds from the combustion of polyethylene and 55 compounds from polypropylene⁹. Wood generates at least as many products¹⁰. The relative contribution of one or various combinations of these combustion products to animal toxicity is so far unknown and would require a major research effort to decipher.

The current awareness of the importance of combustion product toxicity is apparent from two recent symposia (Annual Society of Toxicology meeting, February 1982; American Chemical Society national meeting, March 1982), and by the newly convened combustion product toxicity committees of the US National Fire Protection Association and of the US National Institute of Building Sciences. Recently, three different animal-based test methods designed to assess the acute inhalation toxicity of combustion products were proposed to the Committee on Fire Standards of the American Society for Testing and Materials for their consideration. World-wide concern about the toxicity of combustion products is indicated by increased activity in a Subcommittee on Toxic Hazards of the Fire Test Committee of the

International Organization for Standardization and the development of separate tests in Germany, France, Japan and Belgium.

All these tests provide information on the acute toxicity resulting from the inhalation of combustion products, but each test is performed in different laboratory decomposition conditions. The tests do not address the problem of the total toxic hazard which a fire produces or the contribution of a material (or product in its actual end use) to that hazard under a 'real fire' situation. Nonetheless, some building code officials and state regulators are anxious to evaluate the combustion product toxicity of materials and are examining these test methods with that end in mind. It is important, however, that the acute toxicity (the harmful effects measured by the response of laboratory test animals to a single short exposure to combustion products generated by the thermal degradation of materials in specified conditions) be distinguished from the toxic hazard (the materials' properties and the environmental conditions which increase the probability that a toxic atmosphere will occur and an injury will result). Since any one fire is a dynamic, changing condition and no two fires are alike, no one test can duplicate all 'real fires'. Typical fire degradation modes (smouldering, radiant heat, non-flaming and flaming) can be reproduced in the laboratory, however, and materials can be tested in these conditions. The resultant laboratory toxicity data and other needed information, such as the projected end use of the product, the quantity of material present, its ignition and combustion properties, ventilation conditions, and the density and sensory-irritant properties of the smoke, may then be related to the total toxic hazard. Many of the investigators involved in this area of research are now turning their talents towards the more challenging field of toxic hazard analysis. □

1. *Highlights of Fire in the United States* 2nd edn (Federal Emergency Management Agency, US Fire Administration, 1981).
2. *Preview, Residential Fires in the United States* (Federal Emergency Management Agency, US Fire Administration, 1979).
3. Birky, M.M. *et al.* *Fire and Materials* 3, 211 (1979).
4. Gregory, K.L., Malinoski, V.F. & Sharp, C.R. *Arch. environ. Hlth* 18, 508 (1969).
5. Woolley, W.D. *J. Macromolec. Sci.-Chem.* A17, 1 (1982).
6. *Clark County Fire Department Report on the MGM Grand Hotel in Las Vegas, Nevada, on November 21, 1980*.
7. Petajan, J.H. *et al.* *Science* 187, 742 (1975).
8. Levin, B.C. *et al.* *Further Development of a Test Method for the Assessment of the Acute Inhalation Toxicity of Combustion Products* (NBSIR 82-2532, 1982).
9. Michal, J., Mitera, J. & Tardon, S. *Fire and Materials* 1, 160 (1976).
10. Alger, R. in *The Mechanisms of Pyrolysis, Oxidation and Burning of Organic Materials* (ed. Wall, L.A.) (NBS spec. Pub. 357, Washington DC, 1972).

Erratum

In the *News and Views* article 'Promiscuous DNA — chloroplast genes inside plant mitochondria' by John Ellis (*Nature* 299, 678; 1982), the word 'within' in the sentence 'Most polypeptides found in the bioenergetic organelles are encoded and synthesized within the organelles themselves' should be replaced by the word 'outside'.

Barbara Chernov Levin is Head of the Fire Toxicology Research Center for Fire Research, National Bureau of Standards, Washington, DC 20234.

REVIEW ARTICLE

Genetic and molecular mechanisms of viral pathogenesis: implications for prevention and treatment

Bernard N. Fields & Mark I. Greene

Departments of Microbiology and Pathology, Harvard Medical School and Department of Medicine (Infectious Disease), Brigham and Women's Hospital, Boston, Massachusetts 02115, USA

The pathogenesis of infection of mice by the mammalian reoviruses involves several discrete steps. Each of the three viral outer capsid proteins has a highly distinct and specialized role: one protein ($\sigma 1$) binds to cell surface receptors; a second protein ($\mu 1C$) determines the capacity for viral growth at mucosal surfaces; and the third protein ($\sigma 3$) is responsible for inhibiting cell macromolecular synthesis. A detailed picture of the molecular basis of reovirus virulence and attenuation is now emerging.

TECHNOLOGICAL developments over the past decade have enhanced our understanding of the biochemical basis of heredity and have led to an increasing understanding of the organization and regulation of eukaryotic genes. Although microbial systems have provided many of the models for these basic studies, the complex nature of the interactions between microbial agents and eukaryotic hosts that occur during the infectious process have hindered the deciphering of the molecular mechanisms involved in infectious diseases¹. Thus, while many elegant studies have been performed on the descriptive aspects of the pathogenesis of infectious diseases, little is known about the precise molecular events responsible². Recently, increased knowledge of the functions of individual components of viruses and bacteria and their interactions in the ultimate production of infection have begun to allow research directed towards defining molecular mechanisms of pathogenesis^{1,3,4}.

Here we review studies using the mammalian reoviruses as a model of microbial pathogenesis. We shall describe the role of various viral components in the pathogenesis of reoviral infection of young mice, and illustrate several specific genetic and immunological manipulations which yield attenuated viruses. One of the major goals of studying viral pathogenesis with model systems such as the reoviruses is to understand the processes of attenuation and immunization of microbial pathogens and to develop new methods for the prevention and treatment of infectious diseases.

Reovirus structure and morphology

Genome structure. The mammalian reoviruses include three serotypes⁵. The viral genome consists of three size classes of segmented double-stranded RNA (dsRNA): large (L), medium (M) and small (S), each of which consists of unique segments³. L contains three genome segments (L1–L3) with molecular weights of $\sim 2.3\text{--}2.5 \times 10^6$; M consists of three genome segments (M1–M3) with molecular weights of $\sim 1.4\text{--}1.6 \times 10^6$; S has four genome segments (S1–S4) with molecular weights of $\sim 0.6\text{--}0.9 \times 10^6$. Each of the 10 genome segments represents a single gene which is transcribed and translated into a unique mRNA molecule and a primary polypeptide, respectively^{6–9}.

Virion polypeptides. Solubilization and separation by polyacrylamide gel electrophoresis of the proteins in cells infected with reovirus yields three size classes: large or λ ($\lambda 1$, $\lambda 2$, $\lambda 3$), middle or μ ($\mu 1/\mu 1C$, $\mu 2$, μNS), and small or σ ($\sigma 1$, $\sigma 2$, σNS , $\sigma 3$)⁷. Nine of the polypeptides are present in purified virions ($\lambda 1$, $\lambda 2$, $\lambda 3$, $\mu 1/\mu 1C$, $\mu 2$, $\sigma 1$, $\sigma 2$ and $\sigma 3$) while two are found

exclusively in the cytoplasm (μNS , σNS)^{8,9}. The $\mu 1C$ polypeptide is derived by cleavage from the primary $\mu 1$ polypeptide⁵. The correspondence between genes and proteins is shown in Table 1.

Morphology. Reovirions are icosahedral virions comprising a double capsid(s), which consists of a core containing the 10 genome dsRNA segments and a closely applied inner protein shell surrounded by an outer protein layer. The polypeptides in the core include $\lambda 1$ – $\lambda 3$, $\mu 1$, $\mu 2$ and $\sigma 2$ (refs 5, 8). The core contains the viral transcriptase and replicase⁵. The outer capsid shell consists of three polypeptides $\sigma 1$, $\mu 1C$ and $\sigma 3$ (Fig. 1). Protein $\lambda 2$ is exposed on the outside of the virus, and is thought to represent the core spike¹⁰. It is closely associated with $\sigma 1$, the viral haemagglutinin (HA)¹⁰. The two major outer capsid polypeptides are $\mu 1C$ and $\sigma 3$; these are complexed to each other and are found in a 1:2 ratio in the virion⁵.

Role of outer capsid in pathogenesis of reovirus infection

The pattern of virulence of the mammalian reoviruses is determined primarily by the three outer capsid polypeptides ($\mu 1C$, $\sigma 1$ and $\sigma 3$)³, each of which has a unique role. Following entry into the gastrointestinal tract (the presumed natural portal of entry), the $\mu 1C$ protein is digested by host cell proteases. It is this that induces, in part, the cellular immune response¹¹. The $\sigma 1$ protein (the viral haemagglutinin) interacts with receptors on the surface of immune and non-immune lymphocytes and neurones and in this way determines cell and tissue tropism^{12–16}.

Table 1 Correspondence between reovirus dsRNA genes and polypeptides

Gene segment	Polypeptide	Location in virus
L1	$\lambda 3$	Core
L2	$\lambda 2$	Core spike
L3	$\lambda 1$	Core
M1	$\mu 2$	Core
M2	$\mu 1/\mu 1C$	Outer capsid
M3	μNS	Nonstructural
S1	$\sigma 1$	Outer capsid
S2	$\sigma 2$	Core
S3	σNS	Nonstructural
S4	$\sigma 3$	Outer capsid

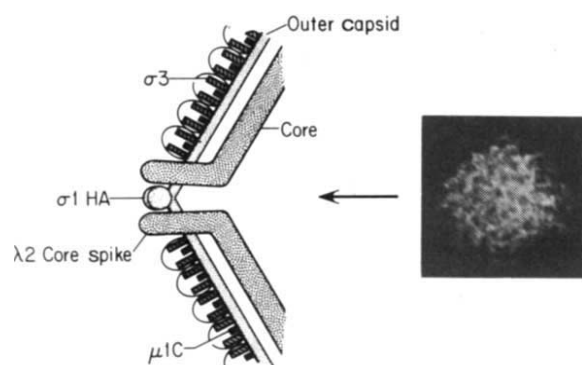


Fig. 1 Schematic diagram of the outer capsid of mammalian reovirus serotype 1, 2, 3. On the right is a negatively stained electron micrograph of an intact virion. On the left is a tentative schematic drawing showing how the three outer capsid proteins ($\sigma 1$, $\sigma 3$ and $\mu 1C$) and the core protein ($\lambda 2$) are organized on the outside of the virion: $\sigma 1$ is located at the vertices of the icosahedron close to $\lambda 2$, the core spike. $\mu 1C$ and $\sigma 3$ are associated with each other on the flat surface of the icosahedron.

It is the major antigen determining specific antiviral humoral or cellular immune responses. The $\sigma 3$ protein is responsible for inhibiting host cell RNA and protein synthesis and has a critical role in allowing the reovirus to initiate persistent infection of cultured cells^{17,18}.

Clearly, the three polypeptides of the outer capsid have different functions. Thus, altering any of them affects the behaviour of reoviruses in distinct ways (Table 2). The relevance of such an alteration to attenuation will now be reviewed in some detail for each protein.

Attenuation of mammalian reoviruses via the viral haemagglutinin ($\sigma 1$)

Allelic substitution. The major function of the haemagglutinin of reovirus in viral pathogenesis is determination of cell and tissue tropism. Intracerebral (i.c.) inoculation of newborn mice with type 3 reovirus leads to the development of an acute encephalitis that is uniformly fatal and associated with specific viral replication in neurones but not in ependymal cells^{19,20}. In contrast, inoculation of type 1 reovirus causes a non-fatal acute ependymitis without involvement of neurones²¹. Localization of the different types of reovirus to different cells is determined in both cases by the $\sigma 1$ protein encoded by the *S1* gene^{16,22}. Thus, substitution of the type 3 *S1* gene by alternative allelic forms of *S1* (from either type 1 or 2) can attenuate the highly virulent type 3 infection by targeting virus to less lethal sites¹⁶. It is important to emphasize that this kind of attenuation is associated with differences in specificity of the immune response of the host. The *S1* gene product is the antigen that evokes the major type-specific humoral and cellular immunological responses¹³⁻¹⁵. As immunity is type-specific, an infection with a reovirus containing a type 1 *S1* gene does not result in

protective humoral or cellular immunity for reoviruses having different type 2 or 3 *S1* genes.

Selective mutations in the *S1* gene. To better define the functions of the viral haemagglutinin, we isolated several monoclonal antibodies directed against the $\sigma 1$ polypeptides²³. As reovirus type 3 is 'neurovirulent', we concentrated largely on analysis of this serotype. We discovered several functionally distinct antigenic regions on the haemagglutinin, one of which is the major site recognized by neutralizing antibodies²³. The same site is involved in the recognition of the reovirus type 3 haemagglutinin by cytolytic T lymphocytes (CTL), since the neutralizing monoclonal antibody blocks the interaction between such cells and reovirus²⁵.

As this site on $\sigma 1$ plays such a critical part in recognition of reovirus by the host immune system, we wished to determine whether it also has an important role in the interaction of reovirus with non-immune cells such as neurones. We reasoned that if the neutralization site of the viral haemagglutinin is involved in binding to receptors on the surface of neurones, variants resistant to neutralization by monoclonal antibodies might lose the capacity to bind to, or enter, neurones. When we isolated such variants by collecting those viral clones which grew in the presence of neutralizing monoclonal antibodies²⁴ and inoculated them into suckling mice, they were found to be either avirulent (lethal does for 50% of the population, LD₅₀ > 10) or markedly reduced in virulence and were impaired in their ability to grow in the brains of newborn mice²⁴. In contrast, the parental Dearing reovirus serotype 3, is highly neurovirulent (LD₅₀ ~ 10¹) and grows to high titre in brain tissue. In fact, examination of brains of mice inoculated with these variants shows a tropism restricted to parts of the hippocampus (D. R. Spriggs, unpublished data). These observations indicate that when variants are selected for resistance to antibody directed at the major neutralization region of the viral haemagglutinin, viruses specifically attenuated for lethal central nervous system infection are generated. The most likely explanation for this type of attenuation is an altered interaction of the viral haemagglutinin with neurone receptors. In addition to being less neurovirulent, these variants show an altered interaction with cytolytic T lymphocytes²⁵. Thus, either the identical site on the reovirus type 3 haemagglutinin protein is recognized by a neutralizing monoclonal antibody, cytolytic T lymphocytes and neurones, or the site(s) important for the last two interactions are close enough to that recognized by the antibody to be seriously affected by antibody binding.

Effect of antibodies against receptors that bind viral haemagglutinin. As suggested above, a decisive element in the successful interaction of the haemagglutinin with certain cells is a highly specific cell-associated receptor for the haemagglutinin. Because the receptor on lymphoid and neuronal cells is able to distinguish subtle differences in the structure of various haemagglutinins, it has been possible to develop a panel of xeno- and monoclonal antibodies to the viral receptor²⁶. We reasoned that the antigen-binding site of antibodies that could interact with the neutralizing area of the haemagglutinin might resemble the binding site of the surface receptor on the neurone.

Table 2 Mutations attenuating reoviral virulence

Gene	Polypeptide	Function	Result of attenuating mutations	Ref.
S1	$\sigma 1$ (HA)	Binds to receptors: I nerve cells II lymphoid cells	Loss of neurovirulence Altered specificity in cytolytic T cell and humoral responses	24
M2	$\mu 1/\mu 1C$	I Growth at mucosal surfaces II Immune induction	Decreased capacity to grow at 1° site Loss of immune stimulation of suppressor T lymphocytes	28 11
S4	$\sigma 3$	III Growth in target tissue Inhibition of RNA and protein synthesis	Relative decrease in neurovirulence Persistent infection Interference	26 16 36
Multiple genes	Many proteins	Pleiotropic	Temperature-sensitive mutations have same tropism but reduced growth <i>in vitro</i> and <i>in vivo</i>	38

The fact that the same determinant recognizes the haemagglutinin suggests that the genetic basis of these binding structures on T lymphocytes or somatic cells (neurons) may involve a gene family similar to that which encodes immunoglobulin variable region products. Alternatively, we considered that limited conformational arrays necessary for HA-specific receptor construction might resemble the conformation of antigen-combining sites on antibodies having the same specificity²⁶.

BALB/c mice were inoculated with the BALB/c-derived monoclonal antibody (MC1) known to interact with the neutralizing region of the haemagglutinin. Spleen cells from these animals were fused to generate a second order panel of hybridomas, which were screened to isolate monoclonal antibody (MC2) potentially capable of interacting with the antigen-binding site of the first monoclonal antibody (John Noseworthy, unpublished). (Previously, we induced antibodies against anti-haemagglutinin by immunizing rabbits with anti-reovirus haemagglutinin antibodies²⁶.) These rabbit anti-anti-haemagglutinin antibodies have been used to mimic viral binding²⁷. More recently, the MC2 antibodies have been found to limit binding of virus to appropriate targets (R. Kaufman, unpublished data). Thus, blocking viral receptors by MC2 prevents viral entry into susceptible cells and consequently the cells may be protected from infection.

The antibodies can also interact with lymphocytes in a manner analogous to the reovirus itself, as judged by the ability of such putative anti-receptor antibodies to activate subsets of T cells and to bind to a panel of cell lines which the virus binds²⁷. We have observed, for example, that MC2 antibodies bind to cells which can interact with reovirus 3. This has been shown for various murine cell types and tumour cell lines of distinct H-2 types, as well as distinct heavy chain allotypes. The YAC (A strain, H-2^a) lymphoma line binds neither reovirus 3 nor MC2, but the R1.1 (C58, H-2^k) thymoma binds both reovirus and the MC2. Absorption of the MC2 with the R1.1 line also absorbs out the activity of MC2 binding to MC1, indicating that MC1-like viral receptors are present on the cell surface. This type of MC2 is not only of value in blocking binding of virus to cells but may be useful also *in vivo*, particularly against local surface infections. As the MC2 resembles a viral antigen, it might also be used to induce or augment immunity to viral diseases without exposure of the host to the virus. This strategy might be most advantageous in the development of an immunogenic 'vaccine', for protection against infection by more highly lethal viruses, for example, the agent responsible for rabies.

Another area of interest is the simple definition of an analogue of virus-receptor interactions by using two monoclonal antibodies, MC1 and MC2: MC1 resembles the receptor and MC2 mimics the virus. It is now possible to assess readily which of several tryptic peptides of the haemagglutinin is capable of inhibiting MC1-MC2 interaction. Those peptides which inhibit this interaction in a radioimmunoassay could probably generate immunity to the most relevant part of the viruses when administered *in vivo*. We predict that this principle (see Fig. 2) should also be applicable to other viruses and may allow the rapid screening and development of new vaccines (synthetic peptides, protein fragments, synthetic compounds). An alternative approach uses reovirus fixed to plates, and radiolabelled MC1 antibody. Tryptic or synthetic peptides of haemagglutinin can then be tested for their ability to inhibit the radiolabelled MC1 binding to the immobilized reovirus. Those peptides capable of inhibiting the interaction would be candidates for immunogens in vaccines. Again, the principle should also extend to other viral systems.

Attenuation via the $\mu 1C$ protein

Effect of route of inoculation on virulence. The Dearing strain of reovirus serotype 3 is highly lethal when inoculated i.c. into suckling mice, a typical LD₅₀ being $\sim 1 \log_{10}$ (ref. 16). However, when this strain is given orally, even in doses of 10^7 plaque-

forming units (PFU) of purified virus, it is avirulent²⁸. This quantity of type 3 reovirus fails to grow in intestinal tissue after it is deposited into the stomach of newborn mice and is inactivated when mixed with intestinal homogenates. In contrast, reovirus type 1 (Lang) strain inoculated by the same route multiplies in the intestine, then spreads to the brain where it produces a benign ependymitis similar to that which develops after intracerebral injection. Another difference between the two viruses is that the infectivity of the type 1 is unchanged or slightly enhanced by treatment with pancreatic protease chymotrypsin while that of the type 3 (Dearing) is dramatically decreased²⁸. A genetic analysis using recombinant viruses generated between types 3 and 1 revealed that the ability to grow in intestinal tissue and the response to pancreatic proteases are properties of the M2 gene and hence the $\mu 1/\mu 1C$ proteins²⁸, but is uncertain whether the two are causally linked.

Effect of variations of M2 on neurovirulence. In addition to determining growth in intestinal tissue, the M2 gene plays a part in controlling the relative degree of neurovirulence of a natural isolate of serotype 3 (ref. 29). A large number of type 3 isolates were screened to ascertain their relative neurovirulence after i.c. inoculation. One strain retained the neurotropism of the type 3 (that is, infection of neurons but not ependyma) but was markedly less virulent (LD₅₀ of $\sim 10^{4.5}$ compared with type 3 Dearing LD₅₀ of $\sim 10^1$). Genetic analysis revealed that the attenuation in neurovirulence in this isolate was also related to its M2 gene²⁹.

The results of these studies indicate that the nature of the M2 gene (and $\mu 1C$ polypeptide) is important in determining virulence after inoculation by the natural oral route and by the experimental intracerebral route. This effect seems to be mediated by how well reovirus grows either at the portal of entry or in the target central nervous system tissue. Thus, while tissue tropism is not affected by the M2 genes, the ability of reovirus to grow and produce a lethal lytic infection is. The manipulation of the M2 gene thus offers the possibility of attenuating reoviruses by reducing virus yield.

Induction of immunity. As indicated previously, the $\mu 1C$ protein is normally sensitive to various proteases present in the

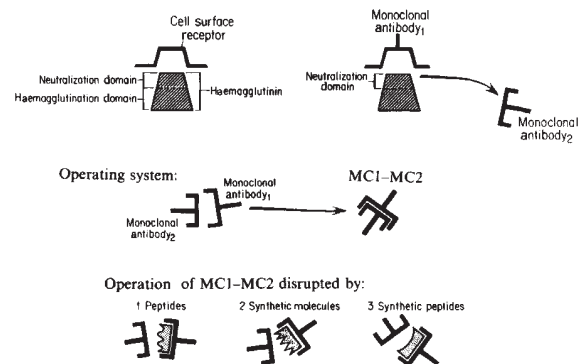


Fig. 2 Definition of an operational analogue of virus-cell receptor interactions. In the reovirus system, the neutralization domain of haemagglutinin interacts with cell surface receptors and the antigen-binding site of the monoclonal antibody (MC1) directed at that domain. A second monoclonal antibody (MC2) made against the antigen-binding site of the first interacts with the cell surface receptor for the neutralization domain, and also interacts with MC1. The MC2-MC1 interaction appears to be the analogue of the virus-cell receptor interaction and constitutes the operating system for studying proteins which can be used as potential immunogens. Only those proteins (or synthetic molecules) that interfere with MC1-MC2 binding are likely to be useful vaccines. In cases where the virus is not dangerous and easily purified, an alternative approach is possible: the virus is immobilized on polystyrene plates, and MC1 antibodies (directed against the domain of the virus that interacts with cell receptors) are radiolabelled and used to bind the immobilized virus. Any peptide or synthetic molecule which limits the binding of the radiolabelled MC1 to the virus is potentially useful as a vaccine. These systems could apply also to bacteria, fungi or parasites^{54,55}.

intestine²⁸. However, oral administration of a virus having a mutant μ 1C protein, resistant to intestinal enzymes, stimulated suppressor T cells (T_s) which abrogated the cellular immune response to the virus¹¹. Thus, the μ 1C polypeptide encoded by the *M2* gene plays a decisive part in the overall pattern of the immunological response when reovirus enters the host by the natural route. One possible explanation of these results is that a virus carrying μ 1C that is resistant to enzymatic cleavage is processed in a manner which stimulates T_s activity. Evidence for special antigen-presenting cells in the generation of T_s has, in fact, recently been demonstrated (Adam Lowy, Jeffrey Drebin and M.I.G., unpublished results).

Attenuation via the σ 3 protein

Effect of high passage on virus properties. Viral vaccines are often prepared by multiple passage of stock virus until attenuation occurs. The critical genetic lesions of most vaccine strains have not been identified. Many viral vaccines are partially temperature-sensitive (ts) suggesting that they may contain multiple mutations³⁰⁻³². As the segmented genome of the reoviruses has allowed us to determine the locus of ts lesions, we investigated the nature of the ts and other lesions produced in reovirus types 1, 2 and 3 after serial, lytic passage³³. Although scoring for every possible mutation was not feasible, several mutations were detected in these stocks, including ts mutants, deletions, cold-sensitive and non-ts interfering lesions. At several stages of passage, most of the ts and non-ts interfering mutants appeared in the tsG group, which has been found on the *S4* gene encoding σ 3 protein³⁴. Thus, after repeated passage of reovirus in tissue culture performed in a manner analogous to generation of attenuated strains, the major mutations appear in the *S4* gene.

Defective interfering particles. Defective interfering (DI) particles are virus particles which lack a portion of the viral genome, contain normal structural proteins, require helper virus to replicate and interfere specifically with replication of homologous standard helper³⁵. Reovirus DI particles are generated after serial passage of ts⁺ or ts mutants in cell culture³⁶⁻³⁸. Reovirus DI particles often appear after several passages with ts⁺ viral stocks, but may be detected earlier in stocks of ts mutants when passage is performed repeatedly. At high multiplicity of infection (MOI), the L1 dsRNA genome segment is most often lost, but deletion mutants lacking segments *L3* and *M1* have also been found. These replicate only in the presence of standard virus, and interfere with the growth of infectious reovirus.

DI viruses are highly attenuated. Because the precise mechanisms involved are unknown, the genetic interactions between reovirus DI and infectious virus were examined³⁹; the results indicate that ts⁺ recombinants form after crosses between reovirus DI particles and ts mutants or ts⁺. We then asked whether DI particles contain other mutations, in addition to the deletion mutation. It was clear that ts and small plaque-low yield mutations are rescued from DI particles; both of these mutations were located on the *S4* gene segment. Indeed, some of the ts and attenuated viruses constructed from these crosses between DI particles and ts were able to interfere with the growth of wild-type reoviruses. Thus, like the repeated passage non-DI stocks, the more defective DI particle stocks were also highly enriched for viruses containing *S4* mutants.

Virus isolates from persistent infection. DI viruses are critical for the establishment of persistent infection in cell cultures⁴⁰. By infecting L cells with DI stocks, a noncytotoxic persistent infection can be established. To determine which gene(s) derived from the DI stock play(s) a critical part in the establishment of persistent infection, cells were co-infected with DI stocks and ts⁺ virus¹⁷. If mutation in a particular gene is essential for the establishment of persistent infection, it should always be derived from the defective virus. Studies designed to examine this hypothesis indicated that the *S4* gene segment was always derived from the defective virus and was critical for attenuation in that it specifically produced non-lytic virus-cell interactions. Interestingly, a major role of the σ 3 protein in the cell during

lytic infection is to inhibit RNA and protein synthesis¹⁸. Mutant σ 3 derived from persistently infected cells, has lost the capacity to do this. Thus, mutations in *S4* are attenuating because they are altered in their ability to lyse cells and inhibit synthesis of cellular protein and RNA. The *in vivo* significance of *S4* mutations has not yet been evaluated.

Temperature-sensitive mutations

That reovirus type 3 Dearing produces a lethal encephalitis after intracerebral inoculation has been discussed above. We have also pointed out that attenuation due to specific lesions in individual viral genes is often associated with viruses capable of full replication in cell cultures. In contrast to this specific type of attenuation, ts mutants whose lesions may be located in one of several viral genes are highly attenuated compared with parental wild-type virus⁴¹. Temperature-sensitive mutants are unable to replicate fully at relatively high temperatures such as the body temperature of the infected host. The extent of the attenuation associated with infection with ts viruses depends in part on the nature of the lesion. However, we now know the location of the lesions used in all our earlier studies and it is clear that attenuation of lethal encephalitis occurs with lesions in the reovirus core as well as with lesions in outer capsid proteins⁴². The attenuation is characterized by an intermediate level of destruction of neurones (that is, 'attenuation') that may be due to a virtually complete block in viral replication (ts C) or a block in replication at more intermediate levels (ts B)⁴¹. In no case have we found evidence of altered tropism with the reovirus ts mutants, although studies using vesicular stomatitis indicate that this may occur⁴³.

Conclusions

For a virus or other microbial agent to produce infection in eukaryotic host it must enter through a major portal, spread (if it produces systemic disease) and ultimately find a distinct cell and tissue site. The host responds to the pathogen both at the portal of entry as well as systemically. Several stages of pathogenicity are common to all types of infectious agents, from the simplest viruses to complex parasites. Because viruses are the smallest and simplest of the infectious agents, they offer the possibility of establishing general principles that explain molecular mechanisms of microbial pathogenicity.

Several conclusions regarding features of attenuation of viral virulence and immunization emerge from this review. First, one viral component, the haemagglutinin, is responsible for determining cell and tissue tropism and is a major antigen for the humoral and cellular immune responses. Selection of viruses that are variant in critical regions of the haemagglutinin leads to attenuation. In addition, presumed anti-receptor antibodies generated against monoclonal antibodies directed against the binding site of the haemagglutinin, potentially can be used both to stimulate an immune response against the virus and possibly to block binding of virus at the level of the cell receptor. Such systems as those which we have defined by MC1 and MC2 monoclonal antibodies are analogues of the virus-cell receptor system and should permit the rapid *in vitro* screening of peptides, synthetic peptides and even synthetic compounds for use as vaccines.

Second, a viral component of the outer capsid, the μ 1C protein, has a critical role in determining the ability of reovirus to replicate at the primary site in the gastrointestinal tract and the ultimate nature of the immune response. Thus, this component provides a second site on the surface of reovirus particles that can be altered, resulting in a reduced capacity of the virus to grow and interact with the host at portals of entry. Third, the σ 3 outer capsid polypeptide, the component responsible for inhibiting synthesis of cellular RNA and protein, is the site of frequent mutations that decrease the capacity of reovirus to lyse cells. Such mutations often appear in viral stocks after they have been repeatedly passaged, and are present at high frequency in defective interfering particles. The presence of such *S4* mutations is associated with attenuation in conditions where defective viruses are present.

Are other microorganisms similar to the mammalian reoviruses in the strategies they use in interacting with eukaryotic hosts during infection, and in the ways that the reoviruses can be attenuated? Although our conclusions are based largely on the results of studies using the reoviruses, it is possible that the lessons of the reoviruses will apply to other microorganisms. That different facets of viral virulence are properties of multiple viral genes has also been noted for the influenza viruses^{4,44,45}. Despite the fact that virulence is multigenic and that multiple genes are involved in infection, individual viral genes are probably responsible for different facets of the process. For example, studies with a number of viruses including poliovirus⁴⁶, coxsackie virus⁴⁷, adenovirus⁴⁸, paramyxoviruses⁴⁹, and influenza virus⁵⁰, have emphasized that generally only one of the viral polypeptides is responsible for interacting with host receptors. While most of these investigations have described experiments using cell cultures rather than animal models, it is likely that similar results will be true *in vivo*. Note that, in most cases, it is the viral haemagglutinin that is the protein responsible for receptor interactions. The detailed mechanisms involving subsequent stages of viral infection involve different proteins and generally are not well understood. For example, influenza virus contains a second outer capsid protein, the neuraminidase, thought to have a role in allowing the virus to come into close contact with the target cell and possibly to aid release of virus from infected cells⁵¹. In addition, several viruses contain a fusion

protein that is thought to participate in initiation of infection and expression of infectivity⁵². Clearly, discrete viral proteins, in addition to the haemagglutinin, are responsible for additional stages of infection.

With regard to aspects of the responses of hosts to viruses, an oligoclonal response as typified by the anti-haemagglutinin antibodies for reovirus can also be seen in the immune response to the influenza viral haemagglutinin. Studies by Liu *et al.*⁵³, for example, have clearly identified a variety of idiotypes against overlapping antigenic determinants on B/Lee haemagglutinin. They found both private as well as shared idiotypes and, in some cases, they detected totally cross-reactive idiotypes on monoclonal antibodies generated against a particular antigenic site. Recently, the effect of anti-idiotypic or anti-receptor reagents against other microorganisms have also been noted^{54,55}. Such reagents may help to elucidate the binding structure for a variety of microbial agents and define new strategies of therapy for modulating microbial infection.

Thus, the increased understanding of the mechanisms of microbial pathogenesis that is based on model systems should provide important new approaches for the prevention and treatment of infectious diseases caused by diverse microorganisms.

We thank our colleagues, postdoctoral fellows and students for their contribution to this work, and Sarah Curwood for typing the manuscript. We acknowledge support of NIH grants AI13178, NS16998 and N316998.

- Smith, H., Skehel, J. J. & Turner, M. J. (eds) *The Molecular Basis of Microbial Pathogenicity*, 1-355 (Verlag-Chemie, Basel, 1980).
- Mims, C. A. *The Pathogenesis of Infectious Disease*, 1-246 (Academic, London, 1976).
- Fields, B. N. *Archs Virol.* **71**, 95-107 (1982).
- Rott, R. *Archs Virol.* **59**, 285-298 (1979).
- Joklik, W. K. *Compreh. Virol.* **2**, 297-334 (1974).
- Both, G. W., Lavi, S. & Shatkin, A. J. *Cell* **4**, 173-180 (1975).
- Zweirink, H. J., McDowell, M. J. & Joklik, W. K. *Virology* **45**, 716-723 (1971).
- Mustoe, T. A., Ramig, R. F., Sharpe, A. H. & Fields, B. N. *Virology* **89**, 594-604 (1978).
- McCrae, M. & Joklik, W. K. *Virology* **89**, 578-593 (1978).
- Hayes, E. C., Lee, P. W. K., Miller, S. E. & Joklik, W. K. *Virology* **108**, 147-155 (1981).
- Rubin, D., Weiner, H. L., Fields, B. N. & Greene, M. I. *J. Immun.* **127**, 1697-1701 (1981).
- Weiner, H. L., Ault, K. A. & Fields, B. N. *J. Immun.* **124**, 2143-2148 (1980).
- Weiner, H. L. & Fields, B. N. *J. exp. Med.* **146**, 1303-1310 (1977).
- Weiner, H. L., Greene, M. I. & Fields, B. N. *J. Immun.* **125**, 278-282 (1980).
- Finberg, R., Weiner, H. L., Fields, B. N., Benacerraf, B. & Burakoff, S. J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 442-446 (1979).
- Weiner, H. L., Drayna, D., Averill, D. R. & Fields, B. N. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5744-5748 (1977).
- Ahmed, R. & Fields, B. N. *Cell* **28**, 605-612 (1982).
- Sharpe, A. J. H. & Fields, B. N. *Virology* (in the press).
- Margolis, G., Kilham, L. & Gonatos, N. R. *Lab. Invest.* **24**, 101-109 (1971).
- Raine, C. S. & Fields, B. N. *J. Neuropath. exp. Neurol.* **32**, 19-33 (1973).
- Kilham, L. & Margolis, G. *Lab. Invest.* **21**, 189-198 (1969).
- Weiner, H. L., Powers, M. L. & Fields, B. N. *J. infect. Dis.* **141**, 609-616 (1980).
- Burstein, S., Spriggs, D. & Fields, B. N. *Virology* **117**, 146-155 (1982).
- Spriggs, D. & Fields, B. N. *Nature* **297**, 68-70 (1982).
- Finberg, R., Spriggs, D. S. & Fields, B. N. *J. Immun.* (in the press).
- Nepom, J. T. *et al. J. exp. Med.* **155**, 155-167 (1982).
- Nepom, J. T. *et al. Survey in Immunologic Research* **11**, 255-261 (1982).
- Rubin, D. & Fields, B. N. *J. exp. Med.* **152**, 853-868 (1980).
- Hardy, D. B., Rubin, D. H. & Fields, B. N. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1298-1302 (1982).
- Plotkin, S. A., Farquhar, J., Katz, M. & Ingalls, T. H. *Am. J. Epidem.* **86**, 468-477 (1967).
- Sabin, A. B. & Boulger, L. R. *J. biol. Stand.* **1**, 115-118 (1973).
- Schwartz, A. J. E. *Annals paediat.* **202**, 251-262 (1964).
- Ahmed, R., Chakraborty, P. R. & Fields, B. N. *J. Virol.* **34**, 285-287 (1980).
- Mustoe, T. A., Ramig, R. F., Sharpe, A. H. & Fields, B. N. *Virology* **85**, 545-556 (1978).
- Holland, J. J. *et al. Compreh. Virol.* **16**, 137-192 (1980).
- Ahmed, R. & Graham, A. F. *J. Virol.* **23**, 250-262 (1977).
- Nonoyama, J., Watanabe, Y. & Graham, A. F. *J. Virol.* **6**, 226-236 (1970).
- Schuerch, A. R., Matshushita, I. & Joklik, W. K. *Intervirology* **3**, 36-46 (1974).
- Ahmed, R. & Fields, B. N. *Virology* **111**, 351-363 (1981).
- Holland, J. J. & Levine, A. *Cell* **14**, 447-452 (1978).
- Fields, B. N. *New Engl. J. Med.* **287**, 1026-1033 (1972).
- Fields, B. N. *Curr. Topics Microbiol. Immun.* **91**, 1-24 (1981).
- Del Canto, M. C., Rabinowitz, S. G. & Johnson, T. C. *Br. J. exp. Med. Path.* **57**, 321-335 (1976).
- Kilbourne, E. D. *Prog. med. Virol.* **5**, 79-126 (1963).
- Mayer, U., Schulman, J. L. & Kilbourne, E. D. *J. Virol.* **11**, 272-278 (1973).
- Boulanger, P. & Lonberg-Holm, K. in *Virus Receptors Pt 2* (eds Lonberg-Holm, K. & Philipson, L.) 21-46 (Chapman & Hall, New York, 1981).
- Philipson, L., Beatrice, S. T. & Crowell, R. L. *Virology* **54**, 69-79 (1973).
- Norrbay, E., Marusyk, H. & Hammerskjold, M. L. *Virology* **36**, 477-489 (1969).
- Scheid, A. in *Viral Receptors Pt 2* (eds Lonberg-Holm, K. & Philipson, L.) 47-62 (Chapman & Hall, New York, 1981).
- Wiley, D. C., Wilson, I. A. & Skehel, J. J. *Nature* **289**, 373-378 (1981).
- Bucher, D. & Palese, P. in *The Influenza Viruses and Influenza* (ed. Kilbourne, E.) 83-119 (Academic, New York, 1975).
- Hosaka, V. & Shimizu, K. in *Virus Infection and the Cell Surface* (eds Poste, G. & Nicholson, G.) 129-156 (North-Holland, Amsterdam, 1977).
- Liu, Y. N., Bona, C. A. & Schulman, J. L. *J. exp. Med.* **154**, 1525-1538 (1981).
- Sacks, D. L., Esser, K. M. & Sher, A. *J. exp. Med.* **155**, 1108-1119 (1982).
- Potocnjak, P., Zavala, F., Nussenzweig, R. & Nussenzweig, V. *Science* **215**, 1637-1639 (1982).

ARTICLES

Observations of reverse polarity flux transfer events at the Earth's dayside magnetopause

R. P. Rijnbeek, S. W. H. Cowley & D. J. Southwood

Blackett Laboratory, Imperial College, London SW7 2BZ, UK

C. T. Russell

Institute of Geophysics and Planetary Physics, University of California at Los Angeles, Los Angeles, California 90024, USA

Observations of flux transfer events (FTEs) at the Earth's dayside magnetopause are presented which have plasma and magnetic field signatures reversed in sign from those previously reported. These FTEs are interpreted in terms of localized and transitory reconnection with the spacecraft located south of the reconnection site. They are associated with a previously reported interval of quasi-steady reconnection which is similarly located, indicating that a close physical connection exists between the two processes.

THE occurrence of large-scale plasma convection within the Earth's magnetosphere, driven by the external flow of the solar wind, has long been recognized as being of fundamental significance in determining the properties, structure and dynamics of magnetospheric plasma populations. There has, however, been much controversy concerning the nature of the physical processes which couple the magnetosphere to the external shocked solar wind (magnetosheath) and which initiate the convection cycle by transporting plasma and magnetic flux from the dayside boundary into the geomagnetic tail. The two main possibilities which have been discussed have been a 'viscous-like' interaction associated with wave-particle interactions and plasma diffusion at the magnetopause boundary^{1,2}, and magnetic reconnection, in which direct magnetic interconnection occurs between the solar wind and the magnetospheric flux tubes, resulting in these 'open' tubes being swept downstream into the tail by the magnetosheath flow³. These two types of processes may, however, coexist, and the determination of their relative importance in driving magnetospheric convection is a central problem in magnetospheric physics. Indirect evidence has supported the importance of reconnection but direct confirmatory observations have been lacking, due to the lack of data of sufficiently high time resolution from the dayside boundary regions.

Recently, high resolution plasma and magnetic field measurements made by the ISEE 1 and 2 spacecraft have become available, and have provided the first definitive observations of magnetic reconnection at the dayside boundary. These observations have shown that the reconnection can occur either as a large-scale, quasi-steady process enduring on time scales of at least a few tens of minutes⁴⁻⁶ as originally envisaged by Dungey³, or as a transient process, localized in both temporal and spatial extent. The latter form, termed the 'flux transfer event' (FTE), was first recognized by Russell and Elphic^{7,8} from its characteristic magnetic signature in ISEE magnetosheath data, although apparently related field fluctuations inside the magnetopause boundary had previously been observed in low resolution HEOS 2 data, and described as possible 'flux erosion' events by Haerendel *et al.*⁹. The interpretation of such field phenomena in terms of localized reconnection has been supported by particle observations indicating that a mixture of magnetospheric and magnetosheath plasma occurs in FTE flux tubes both inside and outside the magnetopause¹⁰⁻¹⁶.

Most of the flux transfer events discussed recently were those observed early in the ISEE 1-2 mission in November 1977 when the spacecraft encountered the magnetopause region on the dawn side of the Earth and at relatively high northern magnetic latitudes (20-40°). All of these encounters have been interpreted in terms of a spacecraft location northward of the reconnection region, such that the open FTE flux tubes which were observed were connected to the Northern Hemisphere of the Earth. The signatures associated with the passage of such a tube across the spacecraft may be readily envisaged with the aid of Fig. 1, following Russell and Elphic^{7,8}. This diagram represents a view of the dayside magnetopause as observed from the magnetosheath, and shows the two 'ends' of a magnetosheath flux tube which has become connected across the magnetopause into the northerly directed (vertical) field of the Earth on the other side of the boundary as a result of a localized reconnection event. Due to magnetic tension, the reconnected flux tubes will contract along the magnetopause in the directions indicated by the broad arrows relative to the overlying magnetosheath plasma and field, causing perturbations in the latter as they do so, as illustrated. If we then consider the magnetic perturbations which should be observed by a spacecraft as the northern open tube passes across it, moving roughly in the direction of the broad arrow, then it is clear that the field will point outwards, away from the magnetopause as the open tube approaches, and inwards, towards the Earth as it recedes, while within the tube strong deflections of the field direction towards that of the Earth's field are expected. The overall plasma flow within the open tube should be deflected both to the north and

to the west relative to the ambient magnetosheath flow due to the magnetic forces (the latter direction clearly depending on the sign of the east-west magnetosheath field component). This basic picture is quite consistent with previous FTE observations presented in refs 7, 8, 13 and 17. In particular all have shown the positive to negative signature of field perturbations directed along the outward normal to the magnetopause, and for those cases where a significant flow perturbation is observed it is directed northwards. Not all the FTEs show clear and consistent northward velocity perturbations however, making their identification as Northern Hemisphere open tubes rather less certain in these cases¹⁶.

If we now consider the southerly tube in Fig. 1, again assumed moving roughly in the direction of its broad arrow, then the perturbations expected in both the magnetopause normal field component and in the plasma flow are reversed in sign relative to the northern tube, while deflections of the reconnected flux tube in the plane of the magnetopause should remain the same, that is northward towards the direction of the Earth's field¹⁶. We present here the first observations corresponding to this situation as observed by the ISEE spacecraft.

Observations

Our data were obtained by the ISEE 1 spacecraft during an inbound crossing of the magnetopause region on 9 August 1978 which occurred at a radial distance of $\sim 15R_E$ and at a local time and magnetic latitude of ~ 1650 h and $\sim 2^\circ$ respectively. The spacecraft was thus located in the dusk local time sector and close to the magnetic equator. Plasma and magnetic field data for a 70-min interval spanning the magnetopause region are shown in Fig. 2. The plasma data were obtained by the Los Alamos/MPI fast plasma analyser and were taken from ref. 5, in which further details of the experiment may be found. Figure 2 shows the plasma density N_p (cm^{-3}) and flow speed in the equatorial plane V_p (km s^{-1}), both obtained at 12 s resolution, together with the GSM z (northward) component of the plasma velocity V_z (km s^{-1}) obtained at 48-s resolution. The magnetic field data were obtained from the UCLA fluxgate mag-

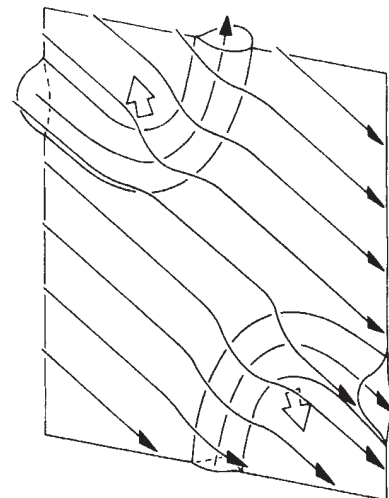


Fig. 1 Sketch of northern and southern 'ends' of open FTE flux tubes shown shortly after reconnection at the dayside magnetopause. The magnetic tension of the open tubes will result in their contraction along the magnetopause relative to overlying magnetosheath plasma and field in the directions indicated by the broad arrows. Consequently the open magnetosheath tubes will tend to tilt towards the direction of the Earth's field (northward) as shown, while the open magnetospheric (boundary layer) tubes will tend to tilt towards the magnetosheath field direction, both north and south of the reconnection site. Perturbations will also occur as shown in the overlying magnetosheath field, particularly in the field component normal to the magnetopause. If the net motion of the open tubes across a spacecraft is roughly as indicated by the broad arrows then the normal field perturbations will be outward followed by inward for the northern tube, and inward followed by outward for the southern tube.

netometer on ISEE 1 (ref. 18), and 12-s field averages plotted every 4 s are shown in boundary normal coordinates. In the latter system $\hat{N}$ is the estimated outward normal to the magnetopause at the spacecraft location and points duskward and sunward nearly in the equatorial plane. $\hat{L}$ is perpendicular to $\hat{N}$ and such that the L - N plane contains the GSM $\hat{z}$ axis; it is directed northward along the magnetopause. $\hat{M}$ completes the orthogonal system and points sunward and dawnward along the magnetopause. In Fig. 2 $\hat{N}$ was determined by assuming that to a sufficient approximation the magnetopause can be treated as a tangential discontinuity, in which the normal magnetic field component threading the current sheet is taken to be zero. The N direction is then given by the cross product of representative field vectors on either side of the boundary, from which its GSM components were found to be (0.753, 0.644, 0.139). This procedure does not really conflict with the possibility that the magnetopause is magnetically open during this traversal as the strength of the normal field is generally expected to be around an order of magnitude less than that of the tangential components. Note also that the above normal is in reasonable agreement with the Fairfield¹⁹ model magnetopause normal for the spacecraft location and also does not differ significantly for our purposes from the minimum variance normal employed by Sonnerup *et al.*⁵. The angle between the Fairfield normal and Sonnerup *et al.* normal and that used here is $\sim 14^\circ$ in both cases.

The basic features of the 9 August 1978 magnetopause passage may be readily discerned from the plasma and field parameters displayed in Fig. 2. Initially the spacecraft is located in the magnetosheath as can be seen from the high plasma density and bulk speed, and from the southward and duskward (tailward) field directions (that is, B_L and B_M are both negative). There then follows an interval from 1934 to 1953 UT which is interpreted by Sonnerup *et al.*⁵ as a partial penetration of the magnetopause/boundary layer region. The magnetosheath is then re-entered, until at 2011 UT a final crossing of the magnetopause occurs after which the magnetic field takes on its northward and sunward (dawnward) magnetospheric direction (that is B_L and B_M are both positive). Inside the magnetosphere after the final magnetopause crossing several pulses of flowing boundary layer plasma also occur.

From a detailed examination of the plasma, energetic particle and magnetic field data Sonnerup *et al.*⁵ were able to deduce that quasi-steady reconnection was taking place in the vicinity of the spacecraft during this magnetopause passage. In particular it was inferred that the magnetopause/boundary layer region observed between 1934 and 1953 UT was located on open magnetic field lines southward of the reconnection region. In Fig. 2 this is indicated by the substantial increases of plasma flow speed observed during this interval, and the southward deflection of its direction. These changes in the plasma flow were shown to be quantitatively consistent with the requirements of tangential stress balance across an open magnetopause with B_N positive (that is, on open tubes connected to the Southern Hemisphere of the Earth). The tangential magnetic field during the 1934 to 1953 UT interval is seen to be intermediate in direction between magnetosheath and final magnetospheric values, that is it is directed predominantly northward and tailward. In the terminology of Hones *et al.*²⁰ this corresponds to an example of 'reverse draping' of the boundary layer field (that is tailward field tilts north of the Equator) on the dusk side of the magnetosphere. In this case, however, the direction of the boundary layer field tilt clearly relates to the direction of the field in the magnetosheath (where B_M is negative) and is just that expected for open tubes being pulled tailward from a reconnection site north of the spacecraft²¹. This observation may help explain the unusually lengthy nature of this apparent 'magnetopause' encounter.

The main feature of the data to which we wish to draw attention here, however, is the occurrence of two prominent FTEs which occur on either side of the quasi-steady open boundary layer region described above. These can be seen in

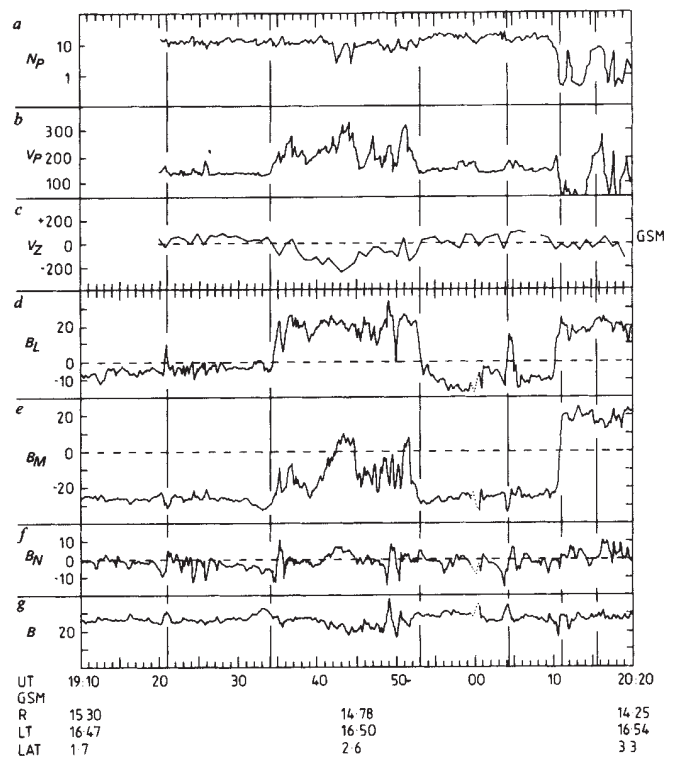


Fig. 2 Plasma and magnetic field data spanning the magnetopause boundary region obtained by ISEE 1 on 9 August 1978. *a-c* Plasma data obtained by the Los Alamos/MPI fast plasma analyser and published by Sonnerup *et al.*⁵. *a* and *b* show the plasma density N_p (cm^{-3}) and equatorial plane bulk speed V_p (km s^{-1}) respectively, both obtained at 12 s resolution while *c* shows the GSM z (northward) component of the plasma velocity V_z (km s^{-1}) obtained at 48 s resolution. The magnetic field data in *d-g* are 12 s averages plotted every 4 s and shown in boundary normal coordinates. A ~ 1 min gap in the ISEE 1 magnetic field data centred on ~ 2000 UT has been filled by ISEE 2 data in the figure, as indicated by the small dotted interval. Note that the ISEE 1 and 2 field data are essentially identical throughout this traversal, due to a small spacecraft separation. The separation vector in L, M, N , coordinates from ISEE 1 to ISEE 2 was $\sim 45, 80, 65$ km. The flux transfer events can be seen at ~ 1921 and ~ 2004 UT.

Fig. 2 at ~ 1921 and ~ 2004 UT and they display all the classic FTE properties described by Russell and Elphic^{7,8} and Paschmann *et al.*¹³ (their category C), except for the negative to positive polarity of the B_N signature and the small but clear southward deflections of the magnetosheath plasma flow. These properties are the reverse from those reported for such events in the literature so far. Properties which remain the same as those previously reported include the following:

- (1) The direction of the tangential field is deflected strongly towards that of the Earth's field as indicated by the changes in both B_L and B_M components (the latter satisfying $\Delta B_M/B_M > 0$).
- (2) Both the magnetic pressure and the plasma pressure (see ref. 5) are considerably enhanced during the events.
- (3) The events occur for southward directed magnetosheath fields ($B_L < 0$).

These properties are clearly those anticipated above for the propagation of localized reconnected FTE flux tubes across the spacecraft which are located south of the reconnection line and connected to the Southern Hemisphere of the Earth.

Note that the negative to positive B_N perturbations in these FTEs extend well outside the interval in which the open tube itself is observed, the latter interval being defined by the sharp northward tilt of the field in the plane of the magnetopause. These extended B_N perturbations result from the distortion of the magnetosheath field overlying the open tube as depicted in Fig. 1, and tend to maximize near the open tube boundaries as expected. Figure 2, however, shows that B_N perturbations

are also present within the open tube itself as previously noted for 'normal' polarity FTEs^{13,16}. The appearance of these B_N fields suggests that the open field lines are twisted about the axis of the FTE flux tube, associated with the presence of a distributed field aligned current (FAC) which is flowing along it. The direction of current flow in the present case is deduced to be parallel to the magnetic field from magnetosphere to magnetosheath, compatible with the direction of low-altitude 'region 1' currents in the dusk sector. Similar conclusions apply to previously published 'normal' polarity FTEs in the dawn sector which again show current flow parallel to the magnetic field, but now directed from magnetosheath to magnetosphere as the tubes are connected to the Northern Hemisphere of the Earth¹⁶. In all these cases the sense of the B_N perturbations observed inside the open tube is the same as those occurring outside the open tube which arise from the distortion of the overlying magnetosheath field, thus resulting in a simple overall bipolar B_N signature. In some circumstances the senses of the B_N perturbations arising from these two sources may differ, so that more complex FTE B_N behaviour may then be anticipated.

Note that in principle a 'reverse' negative to positive B_N FTE signature arising from distortion of the overlying magnetosheath field could also be produced at dusk by the northern tube shown in Fig. 1. This could occur when the tailward-directed magnetosheath flow becomes sufficiently rapid near the magnetospheric flanks that such an open tube can be carried predominantly tailward by the flow against the direction indicated by the magnetic tension (the broad arrow in Fig. 1). This is unlikely to be the case at the ISEE location in Fig. 2 as the magnetosheath flow speed and the Alfvén speed are comparable in magnitude ($\sim 150 \text{ km s}^{-1}$). One could then expect to observe a reduction, if not a reversal in the equatorial plane flow, together with a northward deflection in its direction. The observed variations of the flow do not show such behaviour, but rather are in the opposite direction. We therefore feel that the observations indicate that the 'reverse polarity' FTE events which occur at ~ 1921 and ~ 2004 UT in Fig. 2 are connected to the Southern Hemisphere. This conclusion has been confirmed by examination of energetic ($>45 \text{ keV}$) ion data, which show that both FTEs are associated with enhanced fluxes of ions, inferred to be of magnetospheric origin, streaming parallel to the field from the magnetosphere into the magnetosheath. (P. W. Daly, personal communication).

Finally note that magnetic field and plasma fluctuations which seem to be closely related to the above FTEs can be seen in Fig. 2 in the interval ~ 1922 – 1928 UT following the first clear FTE, as the spacecraft enters the magnetopause region at ~ 1935 UT, within the latter region at ~ 1949 UT and in the second magnetosheath interval at ~ 2000 UT. Other events were also observed before and after the interval displayed. In addition, the large pulse of accelerated boundary layer plasma observed at ~ 2015 UT after the final magnetopause crossing appears to be associated with a 'reverse polarity' negative to positive perturbation in B_N , together with a $\sim 20^\circ$ tailward tilt of the boundary layer field lines towards the direction of the magnetosheath field relative to adjacent magnetospheric field directions. This behaviour of the boundary layer field corresponds to that expected for a magnetospheric open FTE flux tube reconnected north of the spacecraft, although the large general level of magnetic fluctuations occurring in the mag-

netospheric boundary in Fig. 2 makes this identification uncertain. The interpretation of pulse-like boundary layer encounters such as this in terms of magnetospheric FTEs has been discussed previously in refs 12, 13, 16 and 22.

Discussion and conclusions

From an analysis of ISEE 1 plasma and magnetic field data Sonnerup *et al.*⁵ have shown that quasi-steady reconnection was occurring at the dusk magnetopause during an inbound crossing of the boundary region on 9 August 1978. They showed that the spacecraft was located south of the reconnection region at this time, the only such example found in the 11 reconnection events studied by the above authors. This example was also the most southerly located of these events, being only $\sim 2^\circ$ N of the magnetic equator compared with $\sim 20^\circ$ to $\sim 40^\circ$ N typical of the other encounters. We have shown that both before and after the ISEE encounter with the quasi-steady open boundary layer region studied by Sonnerup *et al.*⁵ impulsive reconnection was occurring at the magnetopause as evidenced by the observation of flux transfer events in the plasma and magnetic field data. The signatures of these FTEs differ from those previously reported, however, in that the polarity of the B_N field perturbations is from negative to positive rather than vice versa. This, taken together with the small but clear southward deflections of the plasma flow observed during the events and the energetic ions flowing parallel to the field strongly indicates that these open FTE flux tubes were, like the quasi-steady open boundary layer flux tubes, located south of the reconnection region and hence connected to the Southern Hemisphere of the Earth at this time. As regards the actual position of the reconnection region little can be said at present other than that essentially all the available evidence points towards a roughly equatorial location, possibly tilting north-south away from the subsolar region in response to the IMF B_y component (see for example, refs 23–26) to accommodate results such as the above.

It is significant that FTEs with the above characteristics indicative of connection to the Southern Hemisphere should appear in close association with observations of a quasi-steady open boundary layer region which is inferred to be similarly connected. This association strongly suggests that a close link exists between the two observed reconnection phenomena. At least one other quasi-steady reconnection event is also known to be associated with the appearance of a magnetosheath FTE during the crossing of the boundary region^{5,13}. This case was observed on 11 September 1979 near local noon at $\sim 22^\circ$ north magnetic latitude, and is consistent with the reconnection processes being located south of the spacecraft at that time.

It will be interesting to examine other quasi-steady reconnection events for the presence of associated FTEs. The observations reported here indicate that during the spacecraft penetration of the boundary region the reconnection which was occurring evolved from being unsteady to steady and back again in the local time sector sampled. Whether this represents a basically temporal sequence or rather a spatial pattern which moves into and then out of the spacecraft vicinity remains unclear.

This work was performed while S.W.H.C. was supported by an SERC advanced fellowship, while R.P.R. was supported by a SERC studentship. Work at UCLA was supported by NASA under contract NAS-5-25772.

Received 14 June; accepted 11 August 1982.

- Axford, W. I. & Hines, C. O. *Can. J. Phys.* **39**, 1433–1464 (1961).
- Eastman, T. E. *et al. Geophys. Res. Lett.* **3**, 685–688 (1978).
- Dungey, J. W. *Phys. Rev. Lett.* **6**, 47–48 (1961).
- Paschmann, G. *et al. Nature* **282**, 243–246 (1979).
- Sonnerup, B. U. Ö. *et al. J. geophys. Res.* **86**, 10049–10067 (1981).
- Gosling, J. T. *et al. J. geophys. Res.* **87**, 2147–2158 (1982).
- Russell, C. T. & Elphic, R. C. *Space Sci. Rev.* **22**, 681–715 (1978).
- Russell, C. T. & Elphic, R. C. *Geophys. Res. Lett.* **6**, 33–36 (1979).
- Haerendel, G., Paschmann, G., Scokpe, N., Rosenbauer, H., & Hedgecock, P. C. *J. geophys. Res.* **83**, 3195–3216 (1978).
- Speiser, T. W., Williams, D. J. & Garcia, H. A. *J. geophys. Res.* **86**, 723–732 (1981).
- Daly, P. W., Williams, D. J., Russell, C. T. & Keppler, E. *J. geophys. Res.* **86**, 1628–1632 (1981).
- Daly, P. W. & Keppler, E. *Planet. Space Sci.* **30**, 331–337 (1982).
- Paschmann, G. *et al. J. geophys. Res.* **87**, 2159–2168 (1982).

- Scholer, M., Hovestadt, D., Ipavich, F. M. & Gloeckler, G. *J. geophys. Res.* **87**, 2169–2175 (1982).
- Speiser, T. W. & Williams, D. J. *J. geophys. Res.* **87**, 2177–2186 (1982).
- Cowley, S. W. H. *Rev. Geophys. Space Phys.* **20**, 531–565 (1982).
- Elphic, R. C. & Russell, C. T. in *Magnetospheric Boundary Layers* (ed. Battrick, B.) 43–50 (Rep. ESA SP-148, Noordwijk, 1979).
- Russell, C. T. *IEEE Trans. Geosci. Electron* **GE-16**, 239–242 (1978).
- Fairfield, D. H. *J. geophys. Res.* **76**, 6700–6716 (1971).
- Hones, E. W. Jr *et al. Geophys. Res. Lett.* **9**, 523–526 (1982).
- Cowley, S. W. H., Southwood, D. J. & Saunders, M. A. *Planet. Space Sci.* (to be submitted).
- Cowley, S. W. H. *et al. Planet. Space Sci.* (to be submitted).
- Dungey, J. W. in *Geophysics, the Earth's environment* (eds De Witt, C., Hieblot, J. & Lebeau, A.) 503–550 (Gordon and Breach, New York, 1963).
- Stern, D. P. *J. geophys. Res.* **78**, 7292–7305 (1973).
- Cowley, S. W. H. *Radio Sci.* **8**, 903–913 (1973).
- Gonzalez, W. D. & Mozer, F. S., *J. geophys. Res.* **79**, 4186–4194 (1974).

Environmental radioactivity in Cumbria

D. H. Peirson, R. S. Cambray, P. A. Cawse, J. D. Eakins & N. J. Pattenden

Environmental and Medical Sciences Division, AERE Harwell, Oxon OX11 0RA, UK

The distribution of radioactivity discharged from Sellafield in Cumbria (north-west England) has been measured against a background of nuclear weapon fallout, with the object of determining the pathways and transfer mechanisms between various environmental media.

THE natural radioactivity in the environment arises primarily from uranium and thorium, including the series of decay products, and from potassium. Nowadays, the naturally occurring radioactivity is overlain by artificial radioactivity deposited with the debris from more than 30 yr of nuclear weapon testing. Most of this material, having been injected into the stratosphere, is deposited on a global scale according to a recognized latitudinal pattern¹.

Possible emissions by the nuclear power industry, from reactors or processing plants, will have constituents similar to the radioactivity of the nuclear weapon fallout, namely a mixture of the transuranic elements, fission products and nuclides in gaseous form such as tritium and ¹⁴C. To distinguish the emissions from the fallout it would be necessary to measure the former against baselines set by the latter. As an indication of the scale of the effect of nuclear weapon tests, about 500 Ci of Pu had been deposited in the United Kingdom by 1980²: the current contribution of the external radiation from deposited weapon fallout (mainly ¹³⁷Cs) amounts to about 3%

of the natural background². Radionuclides from weapon tests have been measured continuously for nearly three decades: the cumulative deposition has been assessed directly in a more recent programme of soil sampling throughout Britain. Figure 1 shows the deposition countrywide of ¹³⁷Cs from nuclear weapon fallout up to 1977 plotted in contour form³.

To investigate the environment of the Sellafield (Windscale) establishment in Cumbria (north-west England), it is necessary first to measure the distribution of radioactivity in various environmental media. Then it would be possible to study the mechanisms of transfer between these media, for example, between sea and land, within soil and between soil and plants. Because the amounts of radioactivity are so low the sensitivity of measurement of the radionuclides must be much greater than that required for monitoring according to the normal radiological limits. Artificial radioactivity in Cumbria will arise from: fallout from nuclear weapon testing; discharge to air from the Sellafield works; and discharge to sea from the Sellafield pipelines.

Table 1 Cumulative deposition of ¹³⁷Cs and Pu in Cumbria grassland (1978)

Locality	Average annual rain (mm)	Deposition (pCi cm ⁻²)				Ratio ¹³⁷ Cs/ ²³⁹⁺²⁴⁰ Pu (0–15 cm)
		¹³⁷ Cs (0–15 cm)	¹³⁷ Cs (0–30 cm)	²³⁹⁺²⁴⁰ Pu (0–15 cm)	²³⁸ Pu (0–15 cm)	
1 Biggar	940	8.0	15.3	0.28	0.019	29
2 Rampside	1,026	11.2	16.6	0.16	0.009	70
3 Lindal-in-Furness	1,195	12.0	16.6	0.18	0.011	67
4 Flookburgh	1,120	13.1	17.7	0.38	0.030	35
5 Little Annaside	1,094	7.4	9.8	0.30	0.031	25
6 Beckside	1,399	32.1	38.8	0.33	0.006	97
7 Grizebeck	1,650	12.2	19.0	0.24	0.012	51
8 Abbots Reading	1,470	10.4	13.5	0.16	0.006	65
9 Eskmeals	1,094	23.4	33.8	0.41	0.036	57
10 Stainton	1,259	14.9	16.9	0.30	0.010	50
11 Seathwaite	2,020	19.4	20.2	0.30	0.015	65
12 Hawkshead	1,815	13.1	19.5	0.25	0.014	52
13 Calder Bridge	1,084	36.9	41.4	1.12	0.034	33
14 Wasdale	1,570	15.4	22.2	0.83	0.020	19
15 Great Langdale	3,189	13.3	17.9	0.34	0.010	39
16 Ambleside	2,131	17.4	24.7	0.29	0.012	60
17 Kinniside	1,795	12.9	18.4	0.28	0.011	46
18 Ennerdale	2,344	21.7	25.7	0.80	0.020	27
19 Borrowdale	2,469	25.4	33.3	0.41	0.019	62
20 Patterdale	2,431	12.2	14.7	0.34	0.010	36
21 Ullock	1,340	11.5	15.3	0.22	0.004	52
22 Little Broughton	1,800	10.9	13.5	0.24	0.006	45
23 Lonscale	1,820	10.0	16.2	0.23	0.008	44
24 Threlkeld	1,288	7.0	8.5	0.13	0.003	54
25 Maryport	958	9.6	11.2	0.41	0.019	23
26 Cockermouth	1,279	9.7	14.8	0.38	0.015	26
27 Uldale	1,561	10.1	14.4	0.17	0.009	59
28 Hutton Roof	1,669	10.2	13.3	0.18	0.007	57
29 St Bees (Rottington)	953	5.4	9.5	0.30	0.015	18
30 Harrington	923	8.7	11.4	0.22	0.011	40

The uncertainties in analysis are equivalent on average to coefficients of variation of for ¹³⁷Cs 8%, ²³⁹⁺²⁴⁰Pu 4% and ²³⁸Pu 20%.
1 pCi = 10⁻¹² Ci = 37 mBq.

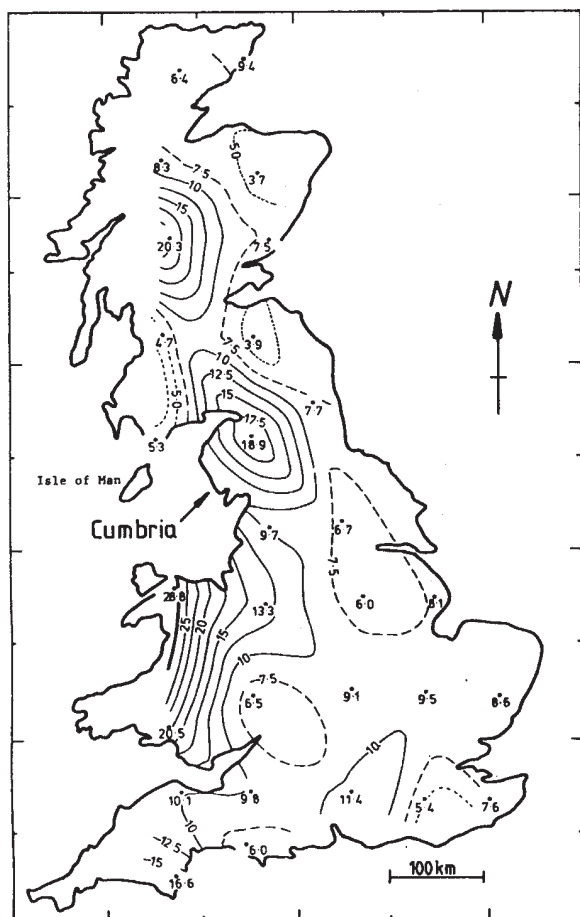


Fig. 1 Cumulative deposition of ^{137}Cs to grassland in Britain (1977). pCi cm^{-2} to 30 cm depth.

The radioactivity discharged by 1978 from Sellafield into the Irish Sea includes more than 12 kCi of Pu α activity and 600 kCi of ^{137}Cs (see ref. 4).

The investigation described here fell into three parts: (1) a survey of radioactive deposits accumulated in soil throughout Cumbria—an intensification of the larger scale survey of Britain; (2) a more detailed survey of soil along the coastal region, together with transects running inland from the coast; (3) measurement of current levels of radionuclides in air and rain.

Cumbria soil survey

The area of some 2,500 km^2 in Cumbria⁵ (and the Isle of Man) was covered by a network of sampling sites based on a grid of 10 km side. Soils were sampled in 1978 to a depth of 30 cm from permanent grassland and woodland. Sites were selected on undisturbed land, that was likely to remain undisturbed to allow repetitive sampling in the future and with no history of flooding.

Samples of soil were taken with a corer of diameter 3.8 cm. At each site at least 10 cores were taken at random at both 0–15 cm and 15–30 cm depth and included the grass and root-mat layer. The cores were bulked to provide composite samples for each depth. The composite samples were air dried (at room temperature) and ground to $<150\text{ }\mu\text{m}$, including stones and roots. About 100 g of soil were analysed for ^{137}Cs and Pu. The content of ^{137}Cs was determined by γ -ray spectrometry using a Ge–Li detector. Plutonium was leached from the soil by a 5:1 mixture of HNO_3/HF using ^{242}Pu as an internal tracer. $^{239+240}\text{Pu}$ and ^{238}Pu were separated from the leachate and analysed by α spectrometry.

The accumulated depositions of ^{137}Cs and Pu are listed in Table 1 in terms of radioactivity per unit area of ground surface.

As mentioned previously these deposits include a contribution from nuclear weapon fallout: the measured deposit relative to weapon fallout is shown in Fig. 2 for the two radionuclides, by converting the results of Table 1 as follows. Average deposition (0–15 cm) from weapon fallout in Britain as determined by the countrywide soil survey is 7.5 pCi cm^{-2} for ^{137}Cs and 0.14 pCi cm^{-2} for $^{239+240}\text{Pu}$ (ref. 3), both normalized to 1,000 mm annual rainfall. (The coefficients of variation are respectively 50 and 25% for 58 sites.) The characteristic ratio in deposition of these radionuclides in 1977 is therefore about 54. Normalization to annual rainfall rests on the effective proportionality between weapon fallout and rain demonstrated in the countrywide survey³, and on earlier work⁶. The average rainfall for the period 1941–70 was extracted from an index⁷ of the rainfall stations nearest to the soil sampling sites. The relative deposition is then given by the ratio of the observed deposit to the nuclear weapon deposit scaled to the mean annual rainfall at the sampling site. Contours of relative rather than absolute deposition are mapped for ease of presentation. The finer structure exhibited by more intensive sampling is not shown. There are uncertainties in the derivation of the relative deposition values, associated with the interpolation between raingauge stations, retention of ^{137}Cs and Pu in the top 15 cm of soil and normal variability in sampling and analysis. Hence only values of relative deposition in excess of 1.5 are considered to be significantly greater than weapon fallout.

Figure 2a shows that the deposition of ^{137}Cs is concentrated near the coast and decreases fairly rapidly inland where the levels are indistinguishable from those due to weapon fallout. The major pattern located around Sellafield represents an accumulation of ^{137}Cs released presumably by aerial emission. To the south-east a smaller peak can be attributed to ^{137}Cs that escaped in the Windscale accident of 1957 (ref. 8). The results for each site (Table 1, Fig. 2) are compounded from the analysis of 10 cores. The relative deposition at site no. 6 of ^{137}Cs is consistent with the results of the Bootle transect⁹.

Figure 2b shows that the contours of relative deposition of $^{239+240}\text{Pu}$ are similarly concentrated around Sellafield, with the hinterland mainly free of excess radioactivity. However, there is no anomaly to the south-east, confirming the absence of measurable amounts of this radionuclide from the emission during the accident in 1957. Another difference from the ^{137}Cs pattern is indicated by the slight enhancement along the coast remote from Sellafield, suggesting that $^{239+240}\text{Pu}$ has arrived from the seaward direction. This indication is supported by the values of the ratio $^{137}\text{Cs}/^{239+240}\text{Pu}$ (Table 1), measured at sampling sites near the coast, that are appreciably lower than the values for the hinterland (which are characteristic of weapon fallout). This implies that there is a relative excess of $^{239+240}\text{Pu}$ along the coast not only in the vicinity of Sellafield but also in the more distant parts. In the Isle of Man no significant deposits in soil of ^{137}Cs and $^{239+240}\text{Pu}$ were found in excess of weapon fallout⁵, because presumably of the greater distance from the point of discharge to the sea.

It is possible to integrate the various depositions to grassland soils in Cumbria over the study area as defined by Fig. 2. Thus the excess of $^{239+240}\text{Pu}$ (to 15 cm depth) due to the Sellafield operation is 2.4 Ci against a background due to weapon fallout of 5.9 Ci: the corresponding estimates for ^{137}Cs are 54 Ci against 315 Ci. The uncertainty of these integrals is probably less than a factor of two.

The radiological implications of these radionuclides in soil may be assessed by considering the potential for resuspension—by wind or other mechanical disturbance—and subsequent inhalation by man. The uppermost layers would be involved in resuspension, whereas most of the deposition over years will have penetrated more deeply into the soil. Calculations of this type have shown that the highest concentrations observed in soil in Cumbria are a very small fraction of the 'derived limit'¹⁵ and hence constitute an insignificant hazard. This limit is derived from the limits set by the International Commission on Radiological Protection for the protection of the general public.

Table 2 Radionuclides in airborne and deposited material (November 1978–October 1979)

	Ratio of coastal station to weapon fallout			Ratio of coastal to inland station			Nuclear* weapon fallout ($10^{-18}\text{Ci kg}^{-1}$)
	St Bees	Eskmeals	Walney	St Bees	Eskmeals	Walney	
Air							
^{137}Cs	9.2	4.9	3.3	0.9	0.8	1.2	240
$^{239+240}\text{Pu}$	12	19	4.0	1.8	2.7	1.9	6
^{241}Am	80	190	30	1.6	9	2.0	0.2
Na (stable)	—	—	—	2.5	1.9	1.3	—
Deposit							($10^{-15}\text{Ci cm}^{-2}$)
^{137}Cs	10.7	8.9	4.7	2.5	2.0	1.0	36
$^{239+240}\text{Pu}$	120	240	56	7.1	14	4.6	0.4
^{241}Am	1,000	1,600	600	10	16	6	0.019
Na (stable)	—	—	—	9.4	5.0	1.7	—

* As observed at Milford Haven¹⁴.

Coastal survey and transects

As well as the comprehensive survey of Cumbrian soil, a more rapid and detailed study was made of the coastal region together with transects running inland from the coast. The coastal and transect surveys⁹ were designed to give an early indication of any radioactive anomalies and also to discover any effect due to the radioactivity discharged from Sellafield into the Irish Sea.

The sampling method was similar to that described above except that the samples were restricted to the top 15 cm of soil. Samples were taken from grassy sites, generally ~2 km apart, from roadside verges, edges of fields and open moorland between December 1977 and March 1978. Six transects were selected, starting at points on the coast between 10 km north (St Bees) and 30 km south (Kirkby) of Sellafield. The transects

extended inland to 20 km: samples were collected at various times between 1977 and 1979. The samples were analysed for ^{137}Cs and Pu isotopes by methods described above: a limited number were also analysed for ^{241}Am .

The results from the 45 central sites in the coastal survey are shown in Fig. 3, together with the ratios $^{137}\text{Cs}/^{239+240}\text{Pu}$ and $^{238}\text{Pu}/^{239+240}\text{Pu}$. The weapon fallout baselines for each parameter are shown as straight lines, because the rainfall is effectively uniform over the coastal region.

Figure 3 shows that there are significant departures from the baseline for all parameters. All sites between 13 km north and 27 km south of Sellafield have excess $^{239+240}\text{Pu}$ over the baseline value (that is >1.5 times). The highest value is 70 times and there are some anomalous values at greater distances. The value

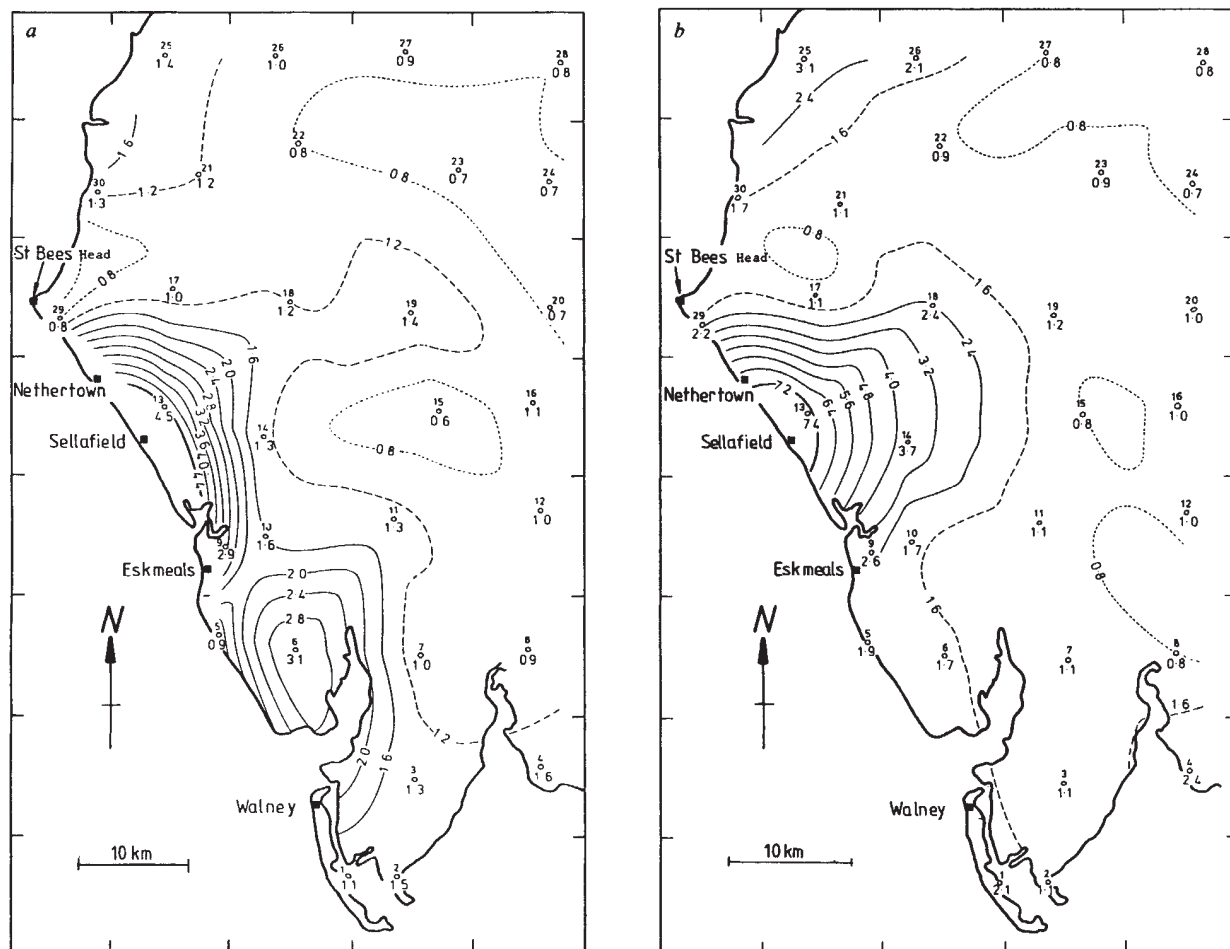


Fig. 2 Cumulative deposition of *a*, ^{137}Cs ; *b*, $^{239+240}\text{Pu}$ in Cumbria grassland (1978) relative to nuclear weapon fallout: 0–15 cm depth. Upper number is site identifier.

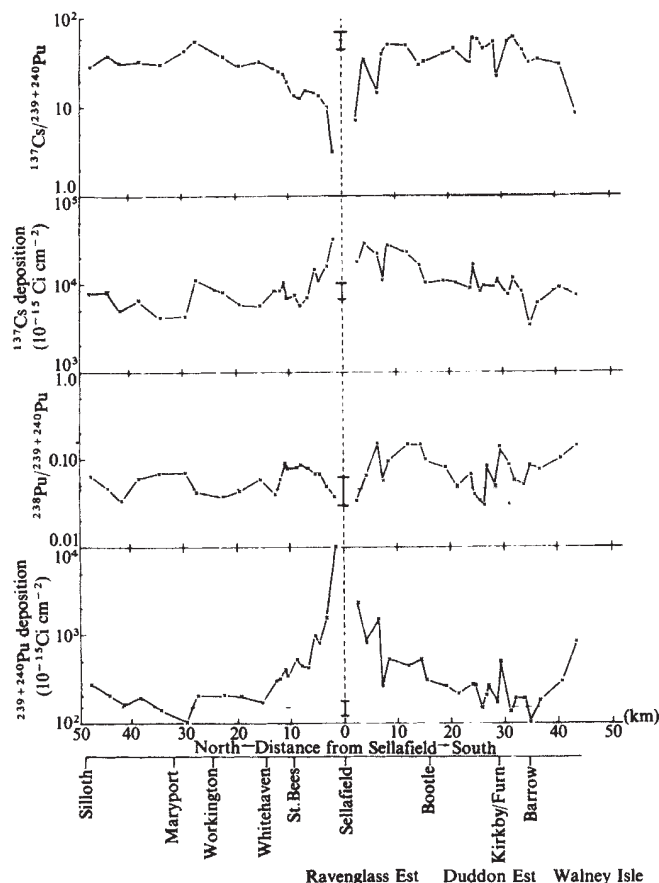


Fig. 3 Cumulative deposition of plutonium and ^{137}Cs in coastal soil of Cumbria (1977-78): 0-15 cm depth. ---, Weapon fallout baseline.

of the ratio $^{238}\text{Pu}/^{239+240}\text{Pu}$ is elevated over the central region except close to Sellafield where it is depressed below the baseline. The deposition of ^{137}Cs does not exceed the baseline by as much as does Pu, in fact throughout the survey the ratio $^{137}\text{Cs}/^{239+240}\text{Pu}$ tends to lie below the baseline. However, the asymmetry of both radionuclides, with more to the south, is greater for ^{137}Cs .

The transect measurements show enhanced values of $^{239+240}\text{Pu}$ at the coast, between 3 and 11 times the weapon fallout baseline^{9,10}. These fall to the baseline value at ~5 km and further inland, and the ratio $^{238}\text{Pu}/^{239+240}\text{Pu}$ is increased within 5 km of the sea above the value 0.05 which is characteristic of weapon fallout¹¹. On the other hand there is no marked enhancement in the deposition of ^{137}Cs along the transects.

The patterns displayed by the coastal and transect surveys may be interpreted as follows. The excess ^{137}Cs can be explained by routine atmospheric discharges from Sellafield, together with the accident in 1957. Some of the excess local plutonium can be attributed to atmospheric discharges but it is difficult to use this source to account for the more remote coastal deposition (in excess of weapon fallout) and in particular for the regular decrease along the transects with increasing distance from the coast. These features suggest a contribution of Pu, but little ^{137}Cs , from the seaward direction. This contribution is marked by an enhanced value for the ratio $^{238}\text{Pu}/^{239+240}\text{Pu}$ approaching the value of ~0.3, characteristic of Sellafield discharges¹², at the coast but merging into the baseline value, of 0.05 due to weapon fallout¹¹, at ~5 km inland. The reduced values of this ratio close to Sellafield can be related to early atmospheric discharges¹³ after short times of irradiation of the fuel, that is before substantial build-up of ^{238}Pu .

A two-dimensional summation of accumulated radionuclides may be based on the coastal and transect results, but neglecting the data from sites within 3 km of Sellafield: these were prob-

ably affected by direct atmospheric discharges. This rough estimate suggests that 1-2 Ci of excess Pu has been deposited in a coastal strip about 5 km wide and 40 km long. The deposit represents 10^{-4} of the quantity discharged to sea⁴.

Airborne and deposited material

So far we have concentrated on an investigation of the cumulative deposition of radionuclides in soil. The coastal survey had shown anomalous accumulation at sites some distance from Sellafield: a complementary series of measurements was made of the current levels of radionuclides in air and rain. Six stations were established in the Cumbrian coastal region, deployed as three pairs, one of a pair on the coast and the other several kilometres inland. The coastal stations were sited at St Bees Head, Eskmeals and Walney (Fig. 2), where an excess of cumulative plutonium had been demonstrated (Fig. 3).

Airborne particulate was sampled continuously at a height of 1 m above ground by passing air through polystyrene filters at the rate of ~75 tonnes per month. Deposited material (wet or dry) was collected in a plastic bottle (25 l), containing tracer and carrier solution, through a funnel of 40 cm diameter. The monthly samples of airborne dust on filters and of rainwater (after evaporation and including the insoluble material) were analysed by γ -ray spectrometry followed by radiochemical methods, whereby Pu and Am were separated, using ^{242}Pu and ^{243}Am as internal tracers, and measured by α spectrometry. Stable trace elements were determined, as appropriate, usually by neutron activation analysis.

The results of these measurements in terms of the annual mean values and monthly ranges for the period November 1978 to October 1979 are reported in detail elsewhere¹⁴ and are available for several radionuclides (actinides and fission products) and stable elements. For the present purpose the data are compressed into Table 2 which is restricted to the results for a few radionuclides and one stable element. These results of concentrations in airborne or deposited material are expressed as ratios to (1) contemporary values of weapon fallout observed at the long-established station at Milford Haven (distance 320 km) and (2) those from the corresponding inland stations. The information in Table 2 demonstrates: (1) an excess of radionuclides compared with nuclear weapon fallout; (2) an excess at the coast compared with inland (except for ^{137}Cs in air); (3) a greater excess for actinides than for fission products (particularly for deposited material); and (4) an apparent difference in the behaviour of airborne and deposited material.

The excess over nuclear weapon fallout is most marked for the actinides in deposited material (Table 2). Most of the additional ^{137}Cs can be explained by contributions from the discharges to air at Sellafield and from seawater that is suspended as spray¹⁴. In the case of actinides the same explanation cannot apply. Most of the actinides come from the seaward direction but if there is an association with suspended seaspray then a large enrichment over normal seawater is required¹⁴, by a mechanism that is under investigation. This required enrichment is indicated by the ratios of the radionuclides to sodium compared with those in bulk seawater from the Irish Sea. The station at St Bees Head is 100 m above sea level and is likely to sample other forms of spray than the other coastal stations at sea level.

The discrepancies between airborne and deposited material in respect of the comparisons with weapon fallout and between coast and inland suggest an instrumental effect due to particle size, based on the postulate that a much larger fraction of the actinides than fission products is carried by relatively large airborne particles. This is qualitatively in accordance with the inference above that the main source of Pu and ^{241}Am is suspension of material coming from the seaward direction, whereas the major contribution to ^{137}Cs (together with ^{134}Cs and some other fission products) comes from the Sellafield discharges to air. In some investigations sea-spray and sand particles have been observed airborne with diameters of 100 μm or greater^{15,16}. The sampler used for collecting airborne particu-

late, like all similar equipment, has a reduced efficiency in field conditions for collecting particles above $\sim 20 \mu\text{m}$ diameter especially in lateral winds of high speed. The same limitation does not apply to the deposition sampler: this explains the discrepancy between the results obtained for airborne and deposited material exhibited in Table 2.

The potential radiological hazard from airborne radioactivity may be estimated by calculating the derived air concentrations for inhalation of $^{239+240}\text{Pu}$, which is the critical radionuclide. (The 'derived air concentration' may be defined by analogy with the definition of the derived limit.) The highest annual average concentration observed was $93 \times 10^{-18} \text{Ci kg}^{-1}$ of air at Eskmeals, representing 0.2% of the derived air concentration¹⁴, modified for the public.

Sea to land transfer

In the general soil survey of Cumbria it was shown (Fig. 2b) that there was a slight but significant enhancement of $^{239+240}\text{Pu}$ in coastal regions remote from Sellafield. This indication is supported by the values (Table 1) of $^{137}\text{Cs}/^{239+240}\text{Pu}$. The ratio is appreciably lower at the coast than that inland, which is characteristic of weapon fallout. This implies an excess of $^{239+240}\text{Pu}$ at the coast and is consistent with a source of the excess lying in a seaward direction. Along the transects running inland the concentration of $^{239+240}\text{Pu}$ was enhanced at the coast and decreased with increasing distance from the coast^{9,10}. This feature suggests a significant contribution of $^{239+240}\text{Pu}$ (and ^{241}Am) but not ^{137}Cs from the sea: the excess is characterized by values at the coast of the ratio $^{238}\text{Pu}/^{239+240}\text{Pu}$ that are higher than the baseline due to nuclear weapon fallout and nearer to that of the Sellafield discharges to sea¹². For the airborne and deposited material the sampling and analysis of current levels at a time of very low values of contemporary weapon fallout

will provide a more sensitive comparison with the baseline than does a comparison of the corresponding accumulations of the radionuclides in soil. In this case the comparison showed an excess of radionuclides at the coast compared with inland (associated with sodium from sea) as well as an excess over nuclear weapon fallout (Table 2). The extra ^{137}Cs could be attributed to sea-spray containing the concentration of seawater from the Irish Sea. For the actinides Pu and ^{241}Am , it is necessary to seek a mechanism of enrichment to explain the higher concentrations relative to sodium.

The radioactivity discharged by 1978 from Sellafield into the Irish Sea includes more than 12 kCi of Pu α activity and 600 kCi of ^{137}Cs (ref. 4). Most of the ^{137}Cs remains in solution whereas about 95% of the plutonium is removed to sediment¹⁷. The evidence summarized above points to a mechanism of physical transfer to land of a very small fraction of the radionuclides in the sea. Possible mechanisms are (1) suspension of the sea surface in the form of spray, either directly by wind or by bubble bursting, (2) injection of seawater and sediment into the air by waves breaking in the surf zone and (3) movement of sediment to intertidal regions and subsequent resuspension by wind. The second possibility, that involves the resuspension of sediment within the sea before release as enriched sea-spray, would satisfy the condition of enrichment that is required for the insoluble $^{239+240}\text{Pu}$ (and perhaps ^{241}Am). In the case of the soluble ^{137}Cs there would be no enrichment. It has been shown that other means of enrichment are possible, for example, of the sea surface microlayer with stable trace elements^{18,19}. More detailed work²⁰ has been started to elucidate the mechanism of sea-to-land transfer of these radionuclides in order to estimate the magnitude of the effect over the long term.

This work was supported in part by British Nuclear Fuels Ltd and the Department of the Environment.

Received 21 June; accepted 20 August 1982.

1. Peirson, D. H. & Cambray, R. S. *Nature* **205**, 433–440 (1965).
2. Cambray, R. S. *et al. Radioactive Fallout in Air and Rain: Results to end of 1981* (AERE-R10485 HMSO, London, 1982).
3. Cawse, P. A., Eakins, J. D., Bocock, K. & Horrill, A. D. *A Survey of Caesium-137 and Plutonium in British Soils (1977)* (AERE-R10155, HMSO, London, in the press).
4. Taylor, F. E. & Webb, G. A. M. *National Radiological Protection Board, Rep. R77* (HMSO, London, 1978).
5. Cawse, P. A. *Studies of Environmental Radioactivity in Cumbria Pt 4* (AERE-R9851, HMSO, London, 1980).
6. Peirson, D. H. & Salmon, L. *Nature* **184**, 1678–1679 (1959).
7. *Rainmaster Dataset* (Meteorological Office, Bracknell, 1979).
8. Dunster, H. J., Howells, H. & Templeton, W. L. *Proc. 2nd Int. Conf. on Peaceful Uses of Atomic Energy* (United Nations, New York, 1958).
9. Eakins, J. D., Pattenden, N. J., Cambray, R. S., Lally, A. E. & Playford, K. *Studies of Environmental Radioactivity in Cumbria Pt 2* (AERE-R9873, HMSO, London, 1981).
10. Cambray, R. S. & Eakins, J. D. *Nature* **300**, 46–48 (1982).

11. Hardy, E. P., Krey, P. W. & Volchok, H. L. *Nature* **241**, 444–445 (1973).
12. *A. Rep. on Radioactive Discharges and Monitoring of the Environment 1979* (British Nuclear Fuels Ltd, 1980).
13. Ellis, F. B., Howells, H., Russell, R. S. & Templeton, W. L. *UKAEA Rep. AHSB(RP) R2* (HMSO, London, 1960).
14. Pattenden, N. J., Cambray, R. S., Playford, K., Eakins, J. D. & Fisher, E. M. R. *Studies of Environmental Radioactivity in Cumbria Pt 3* (AERE-R9857, HMSO, London, 1980).
15. Podzimek, J. & Stampfer, J. F. *Tech. Rep. No. AG-7* (University of Missouri-Rolla, 1977).
16. Gillette, D. & Walker, T. *Soil Sci. Soc. 123*, 97–110 (1977).
17. Hetherington, J. A., Jefferies, D. F. & Lovett, M. B. *Int. Atomic Energy Agency Rep. IAEA-SM-298/29* (1975).
18. Piotrowicz, S. R., Duce, R. A., Fasching, J. L. & Weisel, C. P. *Mar. Chem.* **7**, 307–324 (1979).
19. Pattenden, N. J., Cambray, R. S. & Playford, K. *Geochim. cosmochim. Acta* **45**, 93–100 (1981).
20. Eakins, J. D., Lally, A. E., Burton, P. J., Kilworth, D. R. & Pratley, F. A. *Studies of Environmental Radioactivity in Cumbria Pt 5* (AERE-R10127, HMSO, London, 1982).

Effects of a cloned cell line with NK activity on bone marrow transplants, tumour development and metastasis *in vivo*

John F. Warner & Gunther Dennert

Department of Cancer Biology, The Salk Institute for Biological Studies, PO Box 85800, San Diego, California 92138, USA

Natural killer (NK) cells cloned in vitro have been transferred into NK-deficient hosts. These cells have been shown to have a role in the rejection of allogeneic bone marrow grafts, resistance to both radiation-induced thymic leukaemia and challenge with melanoma tumour cells. It appears that NK cells have an important role in immune surveillance.

THE low incidence of spontaneous tumours in congenitally immunodeficient athymic 'nude' mice¹, which lack functional T cells has raised questions about the role of conventionally induced cytotoxic T lymphocytes (CTL) in the immunosurveillance of tumour formation. Attention has therefore been focused on 'so-called' nonspecific cellular mechanisms such as natural

killer (NK) cells². NK cells, which are present in 'nude' as well as normal mice, can lyse certain tumour cells *in vitro* spontaneously, and therefore may be an important primary line of defence against neoplastic formation *in vivo*³. The low frequency of NK cells in lymphoid organs and the lack of well-defined and unique NK cell surface markers have made

the isolation and study of NK cells rather difficult but recently, murine NK cells have been established and cloned as permanent cell lines *in vitro*⁴. Such NK clones provide a powerful tool with which to explore the *in vivo* functions of these important and interesting cells.

Here we report the *in vivo* effects of an NK cell clone in three experimental animal models. The first involves the rejection of allogeneic bone marrow transplants, the second the inhibition of tumour metastasis, and the third the suppression of radiation-induced thymic leukaemia. First, we show that NK cell-deficient C57BL/6-beige mice^{5,6}, injected with cloned histocompatible NK cells, develop the ability, comparable to that of normal C57BL/6 mice, to reject allogeneic bone marrow grafts. The bone marrow rejection is specific for target structures primarily encoded by genes in the H-2D region of the murine major histocompatibility complex (MHC). Second, using the lung colony assay, we show that lung implantation of intravenously injected B16 melanoma tumour cells in cyclophosphamide-treated (that is, NK-deficient) C57BL/6 mice is markedly inhibited in those mice previously injected with syngeneic NK cells. Third, we demonstrate that the incidence of radiation-induced thymic leukaemia in C57BL/6 mice is dramatically reduced by intravenous administration of syngeneic cloned NK cells 3 months before the normal onset of thymic leukaemia. Taken together, these results support a role for NK cells in bone marrow graft rejection and in the surveillance of neoplasia.

Cloning of NK cells

As C57BL/6 mice were used to study the potential *in vivo* function of NK cells we have established and cloned NK cell lines from C57BL/6 mice for these experiments as previously described for BALB/c and BALB/c *nu/nu* cell clones^{4,7}. C57BL/6 splenocytes were cultured in complete RPMI 1640 medium supplemented with concanavalin A (Con A) conditioned medium⁴. Cell cultures exhibiting NK activity were cloned by limiting dilution and individual isolates assayed for NK activity on a panel of NK-sensitive and resistant tumour targets⁷. The results showed that the cytolytic pattern of these clones was characteristic for NK cells and identical to that previously described for BALB/c NK clones. The cell-surface marker phenotype of the C57BL/6 NK clones was similar to that previously associated with cloned BALB/c NK cell lines⁷,

namely Thy 1.2⁺, T200⁺, Lyt 1⁻, Lyt 2⁻. The presence of dense granules imaged by electron microscopy was also characteristic of these C57BL/6 clones (manuscript in preparation). In the experiments described below, the C57BL/6 cell clone NK B61A2 was used.

Rejection of bone marrow grafts

Previous studies had shown that several positive correlations exist between NK activity *in vitro* and natural resistance *in vivo* to bone marrow transplants⁸. As C57BL/6-beige mice are genetically NK-deficient, they were used to study the potential role of cloned NK cells in the rejection of allogeneic marrow grafts. In a similar model, C57BL/6 mice were made NK-deficient by exposure to four, weekly 175-rad doses of γ -irradiation⁹. Both groups of NK-deficient mice were injected intravenously with 2×10^6 cloned NK B61A2 cells and 7 days later exposed to a lethal dose of γ -irradiation (850–950 rad) followed by transplantation with 2×10^6 allogeneic BALB/c bone marrow cells. Proliferation of donor-derived cells in the spleen (that is, CFU-S formation), indicative of repopulation of the lymphohemoid system, was assessed 5–12 days after marrow transplantation by incorporation into splenocyte DNA of injected ¹²⁵I-labelled 5-iodo-2'-deoxyuridine (¹²⁵IUdR)^{10,11}. Splenic repopulation by transplanted marrow cells was expressed as a percentage of retained radioactivity in the spleen. Results (Table 1a) show that normal C57BL/6 (H-2^b) mice reject BALB/c (H-2^d) bone marrow (that is, <0.05% ¹²⁵IUdR uptake), whereas beige mice and split-dose irradiated C57BL/6 mice are unable to reject allogeneic marrow transplants (that is, ¹²⁵IUdR uptake >0.5% which is equivalent to the syngeneic growth control of BALB/c marrow transplanted into irradiated BALB/c recipients).

In contrast, beige mice and split-dose irradiated C57BL/6 mice, previously injected with H-2 compatible cloned NK B61A2 cells, are capable of rejecting BALB/c bone marrow. Similar results were obtained in experiments assayed 12 days after marrow transplantation. A dose of 2×10^6 NK B61A2 cells was sufficient to cause complete marrow graft rejection in all injected animals when NK cells were administered between 2 and at least 30 days before marrow transplantation. However, when NK cells were administered shortly before marrow transplantation, the percentage of NK-injected mice able to reject marrow grafts was dependent on the dose of NK cells (data not shown).

Table 1 Rejection of allogeneic bone marrow by NK-deficient C57BL/6 mice injected with NK B61A2 cells

<i>a</i>			<i>b</i>											% ¹²⁵ IUdR splenic uptake in irradiated H-2 ^b recipients		
Recipient strain	Bone marrow donor	% ¹²⁵ IUdR splenic uptake		Bone marrow donor H-2 haplotype									<i>bg/bg</i>	<i>bg</i>	<i>bg/bg</i> + NK <i>B61A2</i>	
				K	IA	IB	IJ	IE	IC	S	G	D				
BALB/c	BALB/c	1.36±0.3														
C57BL/6	BALB/c	0.01±0.03	BALB.B	b	b	b	b	b	b	b	b	1.33±0.10	1.13±0.30	1.17±0.05		
C57BL/6 (4x-175r)	BALB/c	0.81±0.04	BALB/c	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	1.73±0.30	0.02±0.01	0.05±0.01		
C57BL/6 (4x-175r)	BALB/c	0.01±0.01	B10	b	b	b	b	b	b	b	b	0.76±0.20	1.57±0.10	1.42±0.30		
+NK B61A2			B10.D2	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	0.49±0.04	0.01±0.001	0.01±0.001		
C57BL/6 <i>bg</i> /+	BALB/c	0.02±0.01	B10.A(18R)	b	b	b	b	b	b	b	<i>d</i>	0.51±0.10	0.01±0.01	0.03±0.01		
C57BL/ <i>bg/bg</i>	BALB/c	1.43±0.1	B10.HTG	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	b	0.62±0.10	0.21±0.01	0.19±0.02		
C57BL/6 <i>bg/bg</i>	BALB/c	0.05±0.01	D2.GD	<i>d</i>	<i>d</i>	<i>d</i>	b	b	b	b	b	0.81±0.10	0.11±0.06	0.19±0.03		
+NK B61A2																

a, Normal C57BL/6, C57BL/6-beige (bg/bg), or C57BL/6 mice treated with four weekly doses of 175 rad γ -irradiation were used as bone marrow graft recipients. Shortly before bone marrow cell transfer, recipient mice were exposed to a lethal dose (850 rad) of ⁶⁰Co γ -irradiation. Donor marrow cells (2×10^6 cells per mouse) were injected intravenously into sex- and age-matched recipients. Proliferation of transplanted cells was assessed 5 days later by measuring the incorporation of ¹²⁵IUdR into splenocyte DNA^{10,11}. Uptake of radioactivity was expressed as a percentage of retained radioactivity in the spleen. The geometric mean and standard error of the arithmetic mean for each group of mice (3–6 animals) are given. Irradiated recipients, syngeneic to the donors, were used as positive controls for uninhibited bone marrow growth (consistently $\geq 0.50\%$ splenic ¹²⁵IUdR uptake). Irradiated mice not injected with marrow cells were used as negative controls for ¹²⁵IUdR incorporation (<0.05% splenic uptake). Groups of mice injected with NK cells received 2×10^6 cloned C57BL/6 NK B61A2 cells intravenously 7 days before bone marrow transplantation. The data presented are from experiments assayed on day 5; however, 12-day assays showed similar results. b, Marrow graft transplantation into C57BL/6-beige and C57BL/6-bg/+ recipients was done as in a, using bone marrow cells obtained from various H-2 recombinant mouse strains possessing *d* alleles at different regions within the H-2 gene complex. Some bg/bg mice were injected intravenously with 2×10^6 cloned NK B61A2 cells 7 days before lethal body irradiation and marrow transplantation (bg/bg + NK B61A2). Marrow growth control values, obtained by injecting donor cells into histocompatible recipients, were comparable to those values obtained by injecting cells from the same preparation into bg/bg recipients. C57BL/6 and C57BL/6 bg/+ recipients showed identical rejection patterns of bone marrow transplants.

Table 2 Effect on allogeneic marrow grafts of NK B61A2 cells and cell lines without NK activity

Recipient	Cell lines used for injection	NK activity of cell lines	Bone marrow donor	% Splenic ¹²⁵ IUdR uptake
a (C57BL/6 × C3H)F ₁	—	—	C57BL/6	0.01 ± 0.001
(C57BL/6 × C3H)F ₁ -bg/bg	—	—	C57BL/6	0.52 ± 0.1
(C57BL/6 × C3H)F ₁ -bg/bg	NK B61A2	+	C57BL/6	0.72 ± 0.1
(C57BL/6 × C3H)F ₁ -bg/bg	BC1	—	C57BL/6	0.95 ± 0.02
(C57BL/6 × C3H)F ₁	—	—	C3H	2.0 ± 0.4
(C57BL/6 × C3H)F ₁ -bg/bg	—	—	C3H	2.7 ± 0.02
(C57BL/6 × C3H)F ₁ -bg/bg	NK B61A2	+	C3H	2.3 ± 0.3
(C57BL/6 × C3H)F ₁ -bg/bg	BC1	—	C3H	2.9 ± 0.6
(C57BL/6 × C3H)F ₁	—	—	BALB/c	0.02 ± 0.002
(C57BL/6 × C3H)F ₁ -bg/bg	—	—	BALB/c	1.69 ± 0.31
(C57BL/6 × C3H)F ₁ -bg/bg	NK B61A2	+	BALB/c	0.21 ± 0.06
(C57BL/6 × C3H)F ₁ -bg/bg	BC1	—	BALB/c	1.93 ± 0.09
b C57BL/6	—	—	BALB/c	0.03 ± 0.01
C57BL/6-bg/bg	—	—	BALB/c	1.42 ± 0.15
C57BL/6-bg/bg	NK B61A2	+	BALB/c	0.02 ± 0.01
C57BL/6-bg/bg	BC1	—	BALB/c	1.05 ± 0.06
C57BL/6-bg/bg	BB1	—	BALB/c	1.46 ± 0.05
C57BL/6-bg/bg	CTLL-2	—	BALB/c	1.33 ± 0.20

Bone marrow transplantation into irradiated normal and beige (C57BL/6 × C3H)F₁ hybrid (a) or C57BL/6 (b) recipients was done as described in Table 1 legend using a 7-day assay. Some groups of mice were injected intravenously 7 days before marrow transplantation with 2×10^6 cells from various cell lines maintained in Con A conditioned medium which either express (+) or do not express (—) NK activity *in vitro* on YAC-1 target cells. Injected cell lines are the following: NK B61A2, a cloned cell line derived from C57BL/6; CTLL-2, a cell line derived from a C57BL/6 anti-BALB/c (H-2^b anti-H-2^d) mixed lymphocyte reaction²⁰; BB1 and BC1, cell lines derived from C57BL/6-beige and (C57BL/6 × C3H)F₁ mice, respectively, by culturing lymphoid cells in Con A conditioned medium. None of the cell lines, except NK B61A2, expresses NK activity. Growth control values for donor bone marrow were equivalent to those obtained in beige recipients and are not included.

As the phenomenon of marrow allograft rejection exhibits a unique specificity for antigens primarily encoded in the H-2D region¹⁰⁻¹³ and to a lesser extent the H-2K region¹⁴ of the MHC, it was important to determine the specificity of marrow graft rejection caused by cloned NK cells. NK cell-injected C57BL/6-beige mice were transplanted with bone marrow obtained from B10 and other congenic mouse strains in order to map the genetic specificity of graft rejection. Results (Table 1b) show that NK-injected beige mice reject BALB/c (H-2^d) but not BALB.B (H-2^b) bone marrow which shows that H-2 encoded target determinants are responsible for rejection. Furthermore, when H-2 recombinant mouse strains are used as bone marrow donors, NK-injected beige mice reject transplanted marrow from donors possessing the H-2D^d allele [B10.A(18R)]. In contrast, relatively weak rejection of marrow cells expressing H-2K end disparity (B10.HTG and D2.GD) is observed¹⁴, whereas H-2 identical bone marrow (B10) is not rejected. Several important conclusions can be drawn from these results: (1) a cloned NK cell line injected into syngeneic NK-deficient mice has an effect *in vivo*; (2) this function is specific in that H-2 disparate, but not H-2 identical bone marrow transplants are rejected; and (3) rejection is primarily, but not exclusively, directed towards antigenic determinants encoded by the H-2D region.

These findings raise the question of whether the effects observed are specifically due to the action of cells expressing NK activity. To demonstrate this, several kinds of controls were done. First, NK B61A2 cells were tested in NK-deficient semi-allogeneic (C57BL/6 × C3H)F₁ beige hosts. Interestingly, in this model, NK B61A2 cells did not cause rejection of either C57BL/6 or C3H marrow transplants, although they do cause a significant but weak rejection of BALB/c allografts (Table 2a). Second, cell lines which do not express NK activity, but require Con A conditioned medium for their growth, do not cause bone marrow rejection. This is shown for several different cell lines. Cell line BC1, obtained from (C57BL/6 × C3H)F₁ mice, grown in Con A conditioned medium and lacking NK activity, when injected into NK-deficient (C57BL/6 × C3H)F₁ beige mice, does not cause rejection of C57BL/6, C3H or allogeneic BALB/c bone marrow (Table 2a). Similarly, cell line BB1, derived from C57BL/6-beige mice, which lacks NK activity, also fails to cause rejection of BALB/c bone marrow grafts when injected into C57BL/6-beige mice (Table 2b). Interestingly, the cell line CTLL-2, derived from a C57BL/6 anti-BALB/c (H-2^b anti H-2^d) mixed lymphocyte reaction²⁰, which also requires Con A conditioned medium for prolifer-

ation and expresses no NK activity, fails to cause bone marrow rejection (Table 2b). These results suggest that bone marrow rejection *in vivo* is caused by cell lines which express NK cytolytic activity *in vitro* and that cell lines merely grown in Con A conditioned medium are not sufficient to cause marrow allograft rejection.

An as yet unexplained observation is that the injection of NK B61A2 cells into (C57BL/6 × C3H)F₁ mice leads to rejection of allogeneic BALB/c but not parental marrow grafts. Perhaps NK cells express exquisite *in vivo* H-2 specificity not obviously demonstrable *in vitro*, as NK B61A2 lyses tumour target cells and peritoneal exudate cells independent of the H-2 haplotype *in vitro* (unpublished results). Accessory cells and/or humoral factors may participate in the rejection of marrow grafts *in vivo*, as one or both components would be absent from NK cytotoxic assays *in vitro*. It is important in this respect that NK B61A2 exhibits antibody-dependent-cell-mediated lysis *in vitro* (unpublished results). Therefore, the

Table 3 Suppression of lung tumour colony formation in NK-deficient mice injected with NK B61A2

Recipient mice	Cell-lines used for injection	NK activity of cell lines	B16/F10 tumour dose	Lung tumour colonies
a C57BL/6	—	—	1×10^5	54 ± 14
C57BL/6-CY	—	—	4×10^5	485 ± 72*
	NK B61A2	+	4×10^5	225 ± 32
C57BL/6-CY	—	—	1×10^5	330 ± 27
	NK B61A2	+	1×10^5	59 ± 18
C57BL/6-CY	—	—	5×10^4	60 ± 11
	NK B61A2	+	5×10^4	12 ± 2
b C57BL/6	—	—	1×10^5	37 ± 16
C57BL/6-CY	—	—	1×10^5	267 ± 12
C57BL/6-CY	NK B61A2	+	1×10^5	43 ± 18
C57BL/6-CY	CTLL-2	—	1×10^5	260 ± 25
C57BL/6-CY	BC1	—	1×10^5	203 ± 2

C57BL/6 mice injected intraperitoneally with cyclophosphamide (CY) (200 mg per kg) 4 days previously, were injected intravenously with various doses of B16/F10 melanoma cells¹⁵. Some mice were also given 2×10^6 cloned NK B61A2 cells, BC1 cells or CTLL-2 cells intravenously 3 h before tumour cell challenge. Pulmonary colonies were counted 3 weeks later after fixation in Bouin's solution and examination under a dissecting microscope. Mice were age- and sex-matched for each experiment. The mean and standard deviation of the number of tumour colonies per lung is presented.

* Mice given 4×10^5 tumour cells alone (a) died before the termination of the experiment so the number of tumour colonies in this group does not show a linear dose relationship. Experiments using C57BL/6-beige mice showed similar results.

Table 4 Decreased incidence of radiation-induced thymic leukaemia in C57BL/6 mice injected with cloned C57BL/6 NK cells

		Cumulative thymoma incidence (mice with tumour/total no. of mice)							
Treatment		Months post-irradiation							
Irradiation	NK	3	4	5	6	7	8	9	% Incidence
—	—	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0
+	—	0/12	3/12	5/12	8/12	10/12	10/12	10/12	83
+	+	0/12	0/12	0/12	1/12	1/12	1/12	1/12	8

Groups of 4-week old C57BL/6 mice were exposed to four weekly doses (175 rad) of ^{60}Co γ -irradiation¹⁹. One week after the final irradiation, mice were injected intravenously with $2-3 \times 10^6$ cloned NK B61A2 cells on a weekly basis for 4 weeks. Mice were observed up to 9 months later, and at death, necropsy was performed to determine the incidence of thymic leukaemia. Presented data are from a representative experiment and show the cumulative number of mice with thymic leukaemia relative to the total number of animals.

failure of NK B61A2 to reject parental marrow grafts as opposed to allogeneic grafts could be a reflection of naturally occurring antibody (that is, anti-allo antibody) in the (C57BL/6 $\times$ C3H)F₁ mice.

An important and related question is whether injection of NK B61A2 cells into NK-deficient mice directly reconstitutes the animal's NK activity or whether other cell types involved in the mechanism of transplant rejection are recruited. Migration studies have shown that only about 1% of the injected NK cells eventually end up in the spleen, a number which is probably too small to be detectable by conventional cytotoxicity assays but may be sufficient to cause bone marrow rejection. Even after 9 days, only low NK activity is detectable in splenocytes of NK-injected mice. This indicates that NK reconstitution is incomplete and would argue against rapid NK cell proliferation or massive recruitment of host NK cells as a result of NK B61A2 injection.

Effect of NK cells on melanoma

Further experiments explored the role of NK cells in the surveillance of neoplasia. Recent studies have implicated NK cells in the control of experimental metastasis of the C57BL/6 melanoma, B16/F10 (refs 15–17). In this model, either C57BL/6-beige or C57BL/6 mice made NK-deficient by prior injection of cyclophosphamide (day 4, 200 mg per kg)¹⁸ are injected with melanoma cells. Tumour-challenged NK-deficient mice develop pulmonary tumour colonies 2–3 weeks later. Using this system, we examined the effect of adoptively transferred cloned NK cells on the development of B16 tumour colonies in the lungs of C57BL/6 mice. Cyclophosphamide-treated mice were injected with 2×10^6 NKB61A2 cells 3 h before intravenous tumour cell injection and the number of melanoma tumour colonies growing in the lungs was assayed 3 weeks later. Results show that mice injected with NK cells exhibit a 50–85% reduction in lung tumour colonies compared with controls (Table 3a). Moreover, tumour colonies were not detected in other organs of NK B61A2 injected mice, whereas control mice frequently showed additional tumours in the liver. In contrast, injection of either CTLL-2 or BC1 cells, which lack NK activity, into NK-deficient mice did not result in a decrease in lung tumour colonies (Table 3b). Experiments using C57BL/6-beige mice showed similar results (data not shown). Hence, injection of cells with NK activity into NK-deficient mice appears to cause inhibition of pulmonary tumour colonies. This demonstrates that cloned NK cells are able to perform a function *in vivo* already suggested by previous studies^{15–17} in which spleen cell preparations high in NK activity were used.

Radiation-induced thymic leukaemia

The *in vivo* suppressive effects caused by this clone of cells with NK activity on B16 melanoma growth led us to investigate the potential antitumour activity of cloned NK cells on the development of radiation-induced thymic leukaemia. In this model, 70–90% of C57BL/6 mice, which are subjected to four weekly doses of 175–200 rad of γ -irradiation beginning at 4 weeks of age, develop lethal thymic leukaemia 3–5 months after the last irradiation¹⁹. Recent studies have shown that this leukaemogenic split-dose irradiation also results in severe depression of NK activity⁹. Therefore, C57BL/6 mice were exposed to leukaemogenic split-dose irradiation and divided into two groups, one of which was given four weekly injections of cloned NK B61A2 cells ($2-3 \times 10^6$ per mouse) after the last irradiation and the other left as control for leukaemia development. Results collected over 9 months show that ~80% of the irradiated control mice developed thymic leukaemia, whereas less than 10% of the irradiated mice injected with syngeneic NK B61A2 cells developed thymoma (Table 4).

We have noted the following observations in experiments currently under way. The lowest incidence of leukaemia occurs in mice injected with NK cells immediately after the last irradiation. Later injection of NK cells (that is 2–3 months after irradiation), at a time when NK activity may slowly reappear, does not seem to prevent development of leukaemia, as is also the case with early injection of cell lines not expressing NK activity. These findings suggest that split-dose irradiated NK-deficient mice, transfused at the proper time with cloned histocompatible NK cells, may become resistant to the development of radiation-induced thymic leukaemia.

Conclusions

Taken together, our observations suggest that NK cells have a crucial role in the mechanism of marrow graft rejection. This in turn may reflect their potential regulatory role in stem cell development and differentiation. Furthermore, NK cells may play an important role in the control of neoplasia. Our experiments, however, do not resolve the question of whether the effects exerted by these cloned NK cells are mediated directly or indirectly. They show, however, the significance of lymphocyte cloning techniques for examining the functional activity of lymphocytes and may point toward experimental approaches in the control of neoplasia.

This work was supported by USPHS grants CA 15581 and CA 19334 (G.D.), the American Cancer Society Grant IM-284 (G.D.) and NRSA Fellowship Award 1 F32 CA/AM 07109-01 (J.W.).

Received 4 August; accepted 13 September 1982.

1. Outzen, H. C., Custer, R. P. & Eaton, G. L. *J. Retic. endothel. Soc.* **17**, 1–9 (1975).
2. Haller, O., Hansson, M., Kiessling, R. & Wigzell, H. *Nature* **270**, 609–611 (1977).
3. Herberman, R. B. & Holden, H. T. *Adv. Cancer Res.* **27**, 305–377 (1978).
4. Dennert, G. *Nature* **287**, 47–48 (1980).
5. Roder, J. & Duwe, A. K. *Nature* **278**, 451–452 (1979).
6. Saxena, R. K., Saxena, Q. B. & Adler, W. H. *Nature* **295**, 240–241 (1982).
7. Dennert, G., Yogeewaran, G. & Yamagata, S. *J. exp. Med.* **153**, 545–556 (1981).
8. Kiessling, R. *et al. Eur. J. Immun.* **7**, 655–663 (1977).
9. Parkinson, D. R., Brightman, R. P. & Waksal, S. D. *J. Immun.* **126**, 1460–1464 (1981).
10. Bennett, M., Cudkovic, G., Foster, R. S. Jr. & Metcalf, D. *J. cell. Physiol.* **71**, 211–236 (1968).

11. Cudkovic, G. & Bennett, M. *J. exp. Med.* **134**, 83–102 (1971).
12. Cudkovic, G. & Lotzova, E. *Transplant Proc.* **4**, 1399–1405 (1973).
13. Cudkovic, G. *Transplant Proc.* **7**, 155–159 (1975).
14. Cudkovic, G. & Warner, J. F. *Immunogenetics* **8**, 13–26 (1979).
15. Hanna, N. & Fidler, I. J. *J. natn. Cancer Inst.* **65**, 801–809 (1980).
16. Talmadge, J. E., Meyers, K. M., Prieur, D. J. & Starkey, J. R. *Nature* **284**, 622–624 (1980).
17. Hanna, N. & Burton, R. C. *J. Immun.* **127**, 1754–1758 (1981).
18. Mantovani, A., Luini, W., Peri, G., Vecchi, A. & Spreafico, F. *J. natn. Cancer Inst.* **61**, 1255–1261 (1978).
19. Kaplan, H. S. & Brown, M. B. *J. natn. Cancer Inst.* **13**, 185–208 (1952).
20. Baker, P. E., Gillis, S. & Smith, K. A. *J. exp. Med.* **149**, 273–278 (1979).

A molecular map of the immune response region from the major histocompatibility complex of the mouse

Michael Steinmetz*, Karyl Minard*, Suzanna Horvath*, Janet McNicholas†, Jeffrey Srelinger‡, Claire Wake§, Eric Long§, Bernard Mach§ & Leroy Hood*

* Division of Biology, California Institute of Technology, Pasadena, California 91125, USA

† Department of Biological Sciences, Stanford University, Stanford, California 94305, USA

‡ Departments of Microbiology and Neurology, University of Southern California School of Medicine, Los Angeles, California 90033, USA

§ Department of Microbiology, University of Geneva Medical School, Geneva, Switzerland

A stretch of 200 kilobases (kb) of DNA from the I region of the mouse major histocompatibility complex has been cloned and characterized. It contains the genes for the biochemically defined class II proteins E_α , E_β and A_β . DNA blot analyses suggest that the I region may contain only 6–8 class II genes. Correlation of our molecular map with the genetic map of the I region confines two of the five I subregions, I-J and I-B, to less than 3.4 kb of DNA at the 3' end of the E_β gene where a hotspot for recombination has been observed. Indeed, the I-A and I-E subregions may be contiguous. If so, the I-B and I-J subregions are not encoded in the I region between the I-A and I-E subregions.

THE major histocompatibility complex (MHC) in mice encompasses about 2 centimorgans of DNA on chromosome 17 and encodes three different classes of proteins involved in immune responses^{1,2}. Class I and class II molecules are both integral cell-surface glycoproteins, whereas class III molecules are serum components of the complement system. Class I molecules, typified by the transplantation antigens, serve as restricting elements for the response of cytotoxic T cells to virally infected cells. Class II molecules, also called *I*-region-associated or Ia antigens, are encoded in the *I* region of the MHC. They regulate the recognition of foreign antigens on the surfaces of macrophages and B cells by T cells³. These interactions lead to high or low immune responses to antigenic determinants controlled by the so-called *Ir* (immune response) genes. The Ia antigens appear to be equivalent to *Ir* gene products⁴. Initially it was believed that the *Ir* genes might encode the presumably vast array of T-cell receptor molecules⁵. However, recent studies using serological and protein analyses have suggested a much more limited number of *Ir* or Ia genes. A genetic map of the MHC is shown in Fig. 1.

The *I* region has been divided by recombinational analysis into five subregions: I-A, I-B, I-J, I-E and I-C⁶. The I-A and I-E subregions contain at least four genes encoding the Ia antigens. The two biochemically well characterized Ia antigens, I-A and I-E, are heterodimers composed of a 33,000 to 35,000-molecular weight α chain and a 28,000 to 31,000-molecular weight β chain and are expressed primarily on B cells and macrophages. The A_α , A_β and E_β chains are encoded in the I-A subregion whereas the E_α peptide is encoded in the I-E subregion. The I-J subregion appears to encode one or more soluble T-cell factors and cell-surface antigens on T cells and macrophages which suppress immune responses. Although these I-J suppressor factors have been serologically defined, their biochemical characterization is only beginning^{7–9}. The I-B and I-C subregions are more controversial^{4,6}. They were originally defined based on their apparent control of certain types of immune responses.

An important characteristic of the MHC is the extreme polymorphism of class I and class II molecules¹⁰. For example, there appear to be over 100 alleles each for the K and D genes of the class I category. Whereas the A_β and E_β loci also are very polymorphic, the A_α and E_α loci exhibit a limited degree of polymorphism. The constellation of alleles present in a particular MHC is called a haplotype. For example, the inbred mouse strain that we have studied, BALB/c, has a *d* haplotype MHC and expresses class I and class II chains denoted K^d , D^d , A_α^d , A_β^d , E_α^d , E_β^d , and so on.

Our initial studies on the genes of the major histocompatibility complex focused on the class I genes^{11–13}. From a cosmid genomic library constructed from BALB/c sperm DNA, we isolated 54 class I clones which could be ordered into 13 clusters spanning 837 kb of DNA and contained 36 distinct class I genes¹⁴. This study demonstrated the effectiveness of cosmid vectors which incorporate very large eukaryotic DNA inserts (~40 kb) in the definition and linkage of MHC genes. Subsequently, we used the techniques of gene transfer to correlate many of these genes with their serologically defined gene products and have initiated studies into the analysis of their functions in cell-cell communication^{15–17}.

To extend this analysis to class II genes in the *I* region, we have used cDNA clones coding for human class II α and β chains^{18,19} and using chromosomal walking procedures, have isolated about 200 kb of contiguous DNA from the *I* region of the inbred BALB/c mouse. Genes for three of the four biochemically defined class II chains, E_α , E_β and A_β , have been identified as has a fourth class II gene, denoted $E_{\beta 2}$, that has not been described previously. Correlation of our molecular map with the genetic map of the *I* region places these genes in the I-A and I-E subregions and confines the I-B and I-J subregions to less than 3.4 kb of DNA at the 3' end of the E_β gene.

cDNA clones for human class II α and β chains

In man, the best known class II antigen is HLA-DR, which exhibits homology for I-E gene products of the mouse^{20,21}. Other loci exist in man for different class II antigens, such as DC²² (probably identical with DS²³) and SB²⁴. It appears that the human HLA-DC antigens are the equivalent of the mouse I-A antigens^{23,25}. Several laboratories have reported the cloning and sequence analysis of cDNA complementary to human messenger RNAs encoding class II α and β chains^{18,19,26–30}. Full-length cDNA clones for the α chain of HLA-DR were isolated from a cDNA library made from the human Raji cell line¹⁸. One of these clones, referred to as DR α , is used here as a probe for the identification of the corresponding mouse genes.

Full-length cDNA clones for the polymorphic β chain of HLA-DR, as well as clones for another 'Ia-like' β chain, clearly distinct from HLA-DR, have been isolated from heterozygous and homozygous human cell lines¹⁹. The genes for these two different class II β chains show a high degree of polymorphism when analysed directly by Southern blot hybridization (C.W. *et al.*, in preparation). Our cDNA clones for this 'Ia-like' β chain are very similar to the β -chain clones reported by

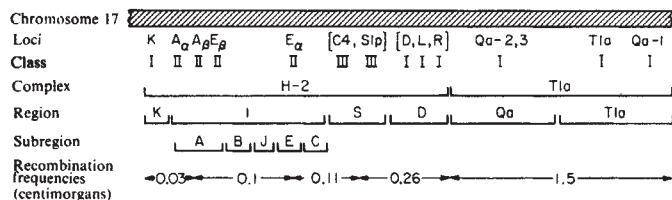


Fig. 1 Genetic map of the major histocompatibility complex of the mouse. The *K*, *D*, *L* and *R* genes encode the transplantation antigens which are found on all somatic cells, whereas the *Qa-2,3*, *Tla* and *Qa-1* genes encode class I molecules which are restricted in their expression to haematopoietic cells. The class III genes *C4* and *Slp* encode C4 components in the *S* region. Genes in brackets have not been mapped with respect to each other. Recombination frequencies are in centimorgans and taken from ref. 2. The centromere is located to the left.

others^{27,29}, and they are all clearly different from HLA-DR β -chain genes^{19,31}. As will be shown here, hybridization of this human 'Ia-like' β -chain gene to mouse DNA shows strongest homology with the *A β* gene. Because the mouse *A α* and human HLA-DC α chains appear homologous^{23,25}, we feel that the 'Ia-like,' non-DR β chain is probably a component of the HLA-DC molecule. In this study, we therefore refer to this β -chain cDNA clone as DC β .

Cloning of 200 kb of DNA from the *I* region

We isolated the mouse *E α* gene by screening a cosmid genomic library previously constructed from BALB/c sperm DNA with the human DR α -chain cDNA clone. This screen yielded the four overlapping cosmid clones 39.1, 17.2, 32.1 and 17.3 which defined a region of about 60 kb of DNA with the *E α* gene in the middle (Fig. 2). We then isolated single-copy probes 4 and 8 from the 5' and 3' ends, respectively, of this 60-kb region and re-screened our cosmid library. Two overlapping clones (24.2 and 8.4) were obtained with probe 4 and eight overlapping clones (57.2, 52.4, 60.3, 21.1, 54.2, 16.2, 47.3 and 55.2) with probe 8. This chromosomal walking procedure was repeated once more with probes 2 and 9 which yielded the cosmid clones 41.1, 29.1 and 7.1 (Fig. 2). Finally, cosmid clone 34.2 was isolated by screening the library with the human DC β -chain cDNA clone.

These 18 overlapping cosmid clones represent about 200 kb of DNA from the *I* region (Fig. 2). Although cosmid clones may delete certain DNA segments¹⁴, there is no evidence for observable deletions since most of the DNA in this 200-kb region is represented by two or more independently generated cosmid clones. Independent evidence for the integrity of the chromosomal map was obtained by Southern blot analyses of BALB/c liver DNA cleaved with four different restriction

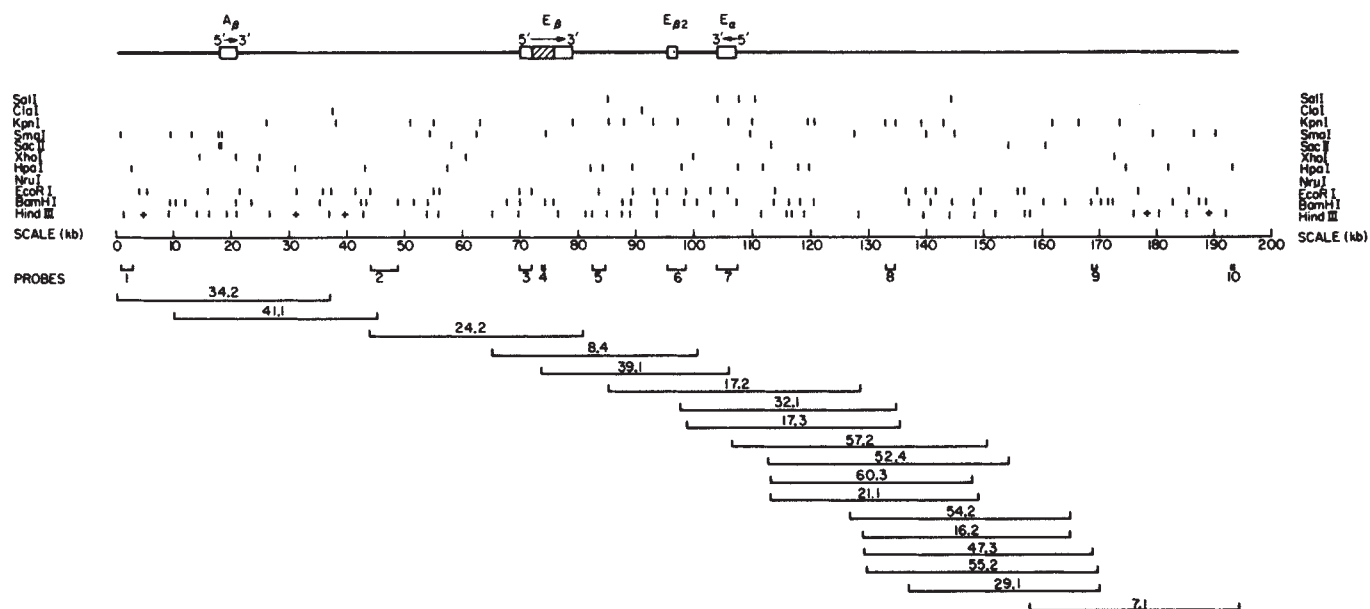


Fig. 2 Molecular map of 200 kb of the *I* region from the BALB/c mouse defined by 18 overlapping cosmid clones and containing four class II genes. Our cosmid BALB/c sperm DNA library, distributed on 66 nitrocellulose filters, contains about 250,000 colonies (representing three genome equivalents)¹⁴. It was hybridized sequentially with the DR α -chain cDNA clone 39-1 prepared from human Raji cell line mRNA¹⁸, probes 4 and 8 from cosmid clones 39.1 and 32.1, probes 2 and 9 from cosmid clones 24.2 and 55.2, and the DC β -chain cDNA clone¹⁹ isolated from Raji cell mRNA. For hybridization the *Pst*I fragments comprising the inserts of DR α and DC β (DR α 39-1: 450 and 650 bp; DC β : 350 and 800 bp in length; corresponding to the 5' and 3' part of the cDNAs, respectively) and the appropriate fragments from the cosmid clones (see below) were isolated by agarose gel electrophoresis and electroelution onto DE81 paper⁴⁴ and labelled by nick-translation to a specific activity of about $1-3 \times 10^8$ c.p.m. μg^{-1} . Hybridization was carried out for 16-18 h at 65 °C with a total of 10^5-10^6 c.p.m. ml^{-1} in $5 \times \text{SSC}$, 10% dextran sulphate, $5 \times$ Denhardt's solution, 5 mM EDTA, 0.1% SDS, $150 \mu\text{g ml}^{-1}$ denatured salmon sperm DNA and $1-2 \mu\text{g ml}^{-1}$ *Hae*III-digested, denatured pBR322 DNA and $50 \mu\text{g ml}^{-1}$ denatured BALB/c DNA to compete for contaminating restriction fragments containing pBR322 or repetitive mouse DNA sequences in the nick-translated probes. The filters were then washed at 65 °C twice for 30-60 min each in $3 \times \text{SSC}$, 0.1% SDS and subsequently treated the same way in $2 \times \text{SSC}$, 0.1% SDS when the screen was with the human cDNA clones, or in $1 \times \text{SSC}$, 0.1% SDS when probes from the cosmid clones were used. Filters were exposed for 1-2 days with intensifying screens, positive colonies were picked from a replica set of the library kept frozen at -80 °C and purified by a second round of plating and hybridization as described previously¹⁴ and above. For the isolation of the 18 overlapping cosmid clones, the same set of filters was used without any treatment between the individual hybridizations. To avoid picking of previously isolated cosmid clones, autoradiograms were compared with those from the last round of hybridization to identify only new cosmid clones. Plasmid DNA was then isolated from the cosmid clones as previously described and mapped with the restriction enzymes indicated by single and double digestions. A + between two restriction sites indicates the presence of more sites in this region for the same enzyme. The location and orientation of the class II genes shown as open boxes was determined by Southern blot analyses of restriction fragments from the cosmid clones with the separated 5' and 3' *Pst*I fragments from the human cDNA clones as hybridization probes. The sizes of the open boxes, therefore, give maximal lengths for the coding regions cross-hybridizing to our human probes. The hatched area in the *E α* gene indicates the location of an intervening sequence presumably between the exons for the β 1 and β 2 domains^{29,37} since the 5' and 3' probes from DC β hybridize exclusively to the left and to the right of the intervening sequence, respectively (the internal *Pst*I site used to generate these probes is located around amino acid residue 90 at the border between the β 1 and β 2 domains). The 10 probes isolated from the 200-kb region were identified as being free of repetitive DNA sequences by their lack of hybridization to total mouse DNA as a labelled probe⁴⁵. They were then shown to be composed of single-copy sequences by Southern blot hybridization against mouse genomic DNA. The 10 probes were isolated by agarose gel electrophoresis⁴⁴. They represent the following restriction fragments: (1) 2-kb *Sma*I-*Hpa*I fragment from clone 34.2; (2) 4.7-kb *Eco*RI-*Bam*HI fragment from clone 24.2; (3) 2-kb *Eco*RI fragment from clone 24.2, subcloned into M13mp8; (4) 500-bp *Sau*3A fragment from clone 39.1 isolated from a 2.7-kb *Bam*HI fragment which extended into vector sequence; (5) 2.3-kb *Hind*III fragment from clone 39.1; (6) 3.2-kb *Eco*RI fragment from clone 8.4; (7) 3.4-kb *Sal*I fragment from clone 32.1, subcloned into M13mp8; (8) 1.6-kb *Kpn*I fragment from clone 32.1; (9) 800-bp *Sau*3A fragment from clone 55.2 isolated from a 2.9-kb *Bam*HI-*Nru*I fragment which extended into vector sequence; (10) 500-bp *Sau*3A fragment from clone 7.1 isolated from a 2-kb *Hpa*I-*Nru*I fragment which extended into vector sequence.

enzymes (*EcoRI*, *BamHI*, *KpnI* and *HindIII*) and hybridized with 10 single-copy probes scattered throughout the 200-kb region (Fig. 2 and below). In all cases the sizes of the genomic fragments agreed with those predicted by the map in Fig. 2. For example, Fig. 3 shows that probe 2 identified unique *EcoRI*, *BamHI*, *KpnI* and *HindIII* fragments in BALB/c DNA of 11, 6, 13 and 11.5 kb, respectively, which are in agreement with the restriction map established by the overlapping cosmid clones 41.1 and 24.2 (Fig. 2). These results confirm the small overlap between the two cosmid clones based on hybridization data and argues against major deletions or rearrangements even in this region, mainly defined by single cosmid clones.

The cloned *I* region contains four class II genes

It was expected that the human DR α -chain cDNA clone would hybridize to the mouse E α gene because of N-terminal amino acid sequence homologies (refs 20, 21 and E. Sung *et al.*, in preparation) and known serological cross-reactions³². The gene identified in the middle of the 200-kb region with the human DR α cDNA clone was subcloned and sequenced. The N-terminal amino acid sequence it encodes is 98% homologous to the E α chain of the *k* haplotype (E. Sung *et al.*, in preparation) and the entire coding region is ~80% homologous to the human DR α sequence at the DNA level³³. We therefore believe that this class II gene is the mouse E α gene and demonstrate below that the E α gene maps into the *I-E* subregion.

As shown in Fig. 2, the E β gene was identified about 30 kb proximal to the E α gene (the order of class II genes appears to be: centromere-A α -A β -E β -E α ^{34,35}). This class II gene was detected by hybridization to a chemically synthesized, 32-fold degenerate oligonucleotide probe 15 nucleotides in length specific for the E β polypeptide. As shown in Fig. 4, this probe hybridized to a 4.8-kb *BamHI* fragment spanning the 5' half of the E β gene (see Fig. 2) in cosmids 24.2 and 8.4. This oligonucleotide probe did not hybridize to any other DNA fragment from the 200 kb of cloned *I* region. The E β gene also cross-hybridizes to the 5' and 3' probes (see below) of the human DC β cDNA clone. As we shall demonstrate later, this class II gene maps to the *I-A* subregion and is the site of crossing-over in the recombinant congenic strain B10.GD as

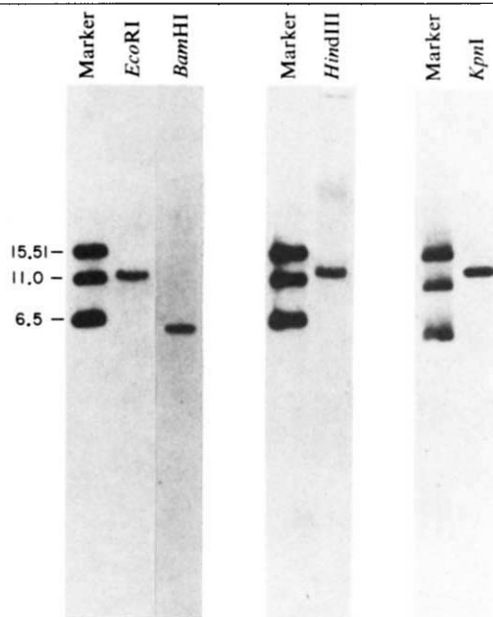


Fig. 3 Southern blot analysis of BALB/c DNA with single-copy probe 2. Ten μ g of BALB/c liver DNA each was digested to completion with the enzymes indicated, separated on a 0.55% agarose gel and transferred to nitrocellulose filters. Hybridization with probe 2 (Fig. 2) was as described in Fig. 2 legend except that cold pBR322 DNA was omitted, probe concentration was 10^6 c.p.m. ml⁻¹, and the washes after hybridization were twice in $3\times$ SSC, 0.1% SDS, and twice in $0.1\times$ SSC, 0.1% SDS for 15 min each at room temperature. Sizes of DNA marker fragments are indicated in kilobase pairs.

predicted from serological and biochemical analyses^{34,36}. Accordingly, we believe we have correctly identified the mouse E β gene.

A second class II β gene was identified between the E β and E α genes by cross-hybridization to the human DC β cDNA clone. This gene hybridizes to the 3' but not to the 5' portion of the human DC β clone. The 5' and 3' probes from DC β were generated by *PstI* digestion which cuts around amino acid

Table 1 Alleles at *H-2* regions and subregions carried by strains of independent origin and *H-2* recombinant strains

Strain	<i>H-2</i> haplotype	Parental <i>H-2</i> haplotypes	Origin of <i>H-2</i> regions							
			<i>K</i>	<i>A</i>	<i>B</i>	<i>J</i>	<i>E</i>	<i>C</i>	<i>S</i>	<i>D</i>
A/WySn	<i>a</i>	(<i>k</i>)/(<i>d</i>)	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	<i>d</i>
B10.RSF-1	<i>an2</i>	<i>at9/f</i>	<i>s</i>	<i>s</i>	.	.	<i>b</i>	(<i>b</i>)	<i>b</i>	<i>f</i>
B10.M(11R)	<i>ap1</i>	<i>f/a</i>	<i>f</i>	<i>f</i>	<i>f</i>	<i>f</i>	<i>f</i>	<i>f</i>	<i>f</i>	<i>d</i>
B10.S(8R)	<i>as1</i>	<i>a/s</i>	<i>k</i>	<i>k</i>	.	.	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>
TBR-2	<i>at2</i>	<i>t1/b</i>	<i>s</i>	<i>k</i>	.	.	<i>b</i>	(<i>b</i>)	<i>b</i>	<i>b</i>
B10.RSB-1	<i>at9</i>	<i>t4/h4</i>	<i>s</i>	<i>s</i>	.	.	<i>b</i>	(<i>b</i>)	<i>b</i>	<i>b</i>
B10.RSB-2	<i>at10</i>	<i>t4/h4</i>	<i>s</i>	<i>s</i>	.	(<i>k</i>)	<i>k</i>	(<i>d</i>)	<i>d</i>	<i>b</i>
B10.RSB-3	<i>at11</i>	<i>t3/h2</i>	<i>s</i>	<i>s</i>	(<i>s</i>)	(<i>s</i>)	<i>k</i>	(<i>d</i>)	<i>k</i>	<i>b</i>
B10.RSB-4	<i>at19</i>	<i>t4/h4</i>	<i>s</i>	<i>s</i>	.	(<i>k</i>)	<i>k</i>	(<i>d</i>)	<i>d</i>	<i>b</i>
B10.RSB-5	<i>at20</i>	<i>t4/pb</i>	<i>s</i>	<i>s</i>	.	(<i>k</i>)	<i>k</i>	.	<i>b</i>	<i>b</i>
C57BL/6	<i>b</i>		<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
B10.F(14R)	<i>bw</i>	<i>b/n</i>	<i>b</i>	<i>b</i>	(<i>b</i>)	(<i>b</i>)	<i>b</i>	.	<i>p</i>	<i>p</i>
BALB/c	<i>d</i>		<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>
B10.D2	<i>d</i>		<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>
B10.GD	<i>g2</i>	<i>d/b</i>	<i>d</i>	<i>d</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
B10.A(4R)	<i>h4</i>	<i>a/b</i>	<i>k</i>	<i>k</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
B10.A(3R)	<i>i3</i>	<i>b/a</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>k</i>	<i>d</i>	<i>d</i>	<i>d</i>
B10.A(5R)	<i>i5</i>	<i>b/a</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	<i>d</i>
C3H	<i>k</i>		<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>
B10.P	<i>p</i>		<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
B10.Q	<i>q</i>		<i>q</i>	<i>q</i>	<i>q</i>	<i>q</i>	<i>q</i>	<i>q</i>	<i>q</i>	<i>q</i>
A.SW	<i>s</i>		<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>
A.TL, B10.TL	<i>t1</i>	<i>s/a1</i>	<i>s</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>d</i>
B10.HTT	<i>t3</i>	<i>s/t1</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>d</i>
B10.S(9R)	<i>t4</i>	<i>s/a</i>	<i>s</i>	<i>s</i>	.	.	<i>k</i>	<i>d</i>	<i>d</i>	<i>d</i>
B10.RPD-1	<i>td1</i>	<i>t2/pb</i>	<i>p</i>	<i>p</i>	(<i>p</i>)	(<i>p</i>)	<i>p</i>	.	<i>b</i>	<i>d</i>
B10.RPD-2	<i>td2</i>	<i>t2/pb</i>	<i>p</i>	<i>p</i>	(<i>p</i>)	(<i>p</i>)	<i>p</i>	.	<i>b</i>	<i>d</i>

Vertical bars indicate presumed locations of crossing-over. Dots are given where the origin of the region is unknown. Parentheses for *H-2* alleles mean that their origin has only been deduced from the composition of the parental haplotypes. Parentheses for the parental haplotypes of strain A indicate that the *a* haplotype has not been derived from a known cross. Strains B10.RSF-1, TBR-2, B10.RSB-1, B10.RSB-2, B10.RSB-3, B10.RSB-4, B10.RSB-5, B10.F(14R), B10.RPD-1 and B10.RPD-2 were from C. David, B10.GD and B10.TL from J. Klein and C3H from the Jackson Laboratories. All other strains were raised in our colony (J.F.).

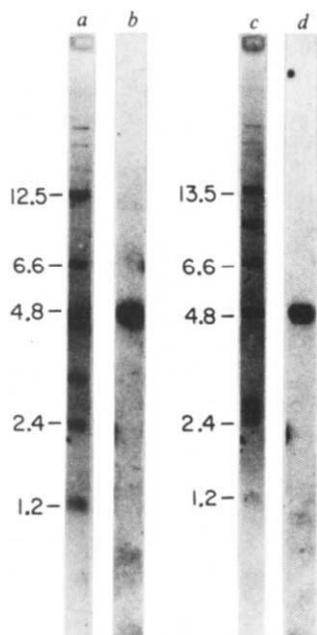


Fig. 4 Identification of the E_β gene in cosmids 8.4 and 24.2 by hybridization to a specific oligonucleotide probe. Cosmid clones 8.4 and 24.2 (0.25 μ g each) were digested with *Bam*HI, separated on a 0.55% agarose gel and transferred to nitrocellulose filters. The hybridization probe, composed of a mixture of 32 15-mer oligonucleotides, was synthesized on polymer support using the phosphoramidite nucleoside chemistry⁴⁶. The heterogeneous nucleotide sequence of the probe was based on the sequence Glu-Cys-His-Phe-Tyr (E. Sung *et al.*, in preparation) for residues 15 to 19 of the E_β chain. The probe was labelled with T4 polynucleotide kinase and [γ -³²P]ATP⁴⁷. Hybridization was at 37 °C in the buffer described in Fig. 2 legend (except that the pBR322 and mouse DNAs were omitted) for 24 h with 5×10^5 c.p.m. ml⁻¹. The filters were washed twice for 15 min at room temperature in $3 \times$ SSC, 0.1% SDS and exposed with intensifying screens overnight. Sizes of DNA fragments are in kilobase pairs. *a* and *c* represent lanes containing ethidium bromide-stained DNA fragments from cosmids 8.4 and 24.2, respectively. *b* and *d* represent lanes containing autoradiograms of *a* and *c*, respectively, after hybridization.

residue 90 thereby separating the coding sequence for the first and second external domains^{19,37}. A genomic fragment encoding the 5' end of the E_β gene (probe 3, Fig. 2) also shows no hybridization to this second β gene. Moreover, this gene (probe 6, Fig. 2) hybridizes to the 3' exons of the E_β gene but not detectably to the A_β gene on genomic blots in stringent conditions. Accordingly, this class II gene is E_β -like and we have denoted it $E_{\beta 2}$. The 5' exons of gene $E_{\beta 2}$ might either be missing or so diversified that they no longer cross-hybridize to the corresponding mouse E_β and human DC_β probes. Thus, $E_{\beta 2}$ might either be a pseudogene or another functional β gene whose product has not yet been identified by serological methods.

About 50 kb proximal to the E_β gene, a third class II β gene was identified with the human DC_β cDNA clone. As we shall see subsequently, this gene maps to the *I-A* subregion and represents the strongest band in a Southern blot analysis of BALB/c DNA with the human DC_β probe. Because this gene is distinct from the other two more closely related E_β -like genes by Southern blot analysis, it seems to be the A_β gene. Direct DNA sequence analysis has confirmed that the N-terminal amino acid sequence it encodes is identical in all positions that can be compared with the A_β protein sequence (M. Malissen, unpublished results).

The class II genes identified in the 200-kb region have several interesting features. First, the orientation of these genes has been determined with probes specific for the 5' and 3' ends of the class II genes isolated from the human DR_α and DC_β cDNAs. The E_α gene has a 5' to 3' orientation that is opposite of that for the A_β and E_β genes (Fig. 2). Second, there is a large intervening sequence in the E_β gene that presumably

separates the exons encoding the two external domains of the β chain (see Fig. 2 legend). Third, the identification of probably three of the four genes encoding serologically defined class II polypeptides, suggests that we have already cloned a major fraction of the *I* region. Direct confirmation that these genes do indeed encode the class II polypeptides will come from gene transfer and expression studies.

The BALB/c genome seems to contain two α and four to six β genes

To estimate the number of class II α and β genes in the mouse genome, we carried out Southern blot analyses in low stringency conditions using as probes the mouse E_α gene and the human DC_β cDNA clone. These data suggest that there is a very limited number of class II genes. Figure 5a shows that the α probe detects the two *Eco*RI fragments of the E_α gene and a third fragment which could represent the A_α gene. Thus there appear to be only two class II α genes in the mouse genome. The human β chain cDNA probe (Fig. 5b) detects the two *Bam*HI fragments comprising the E_β gene and the *Bam*HI fragments containing the A_β and $E_{\beta 2}$ genes. In addition to these cloned β genes, there are two or three extra bands presumably representing one to three additional β genes in the mouse genome. The human cDNA probe hybridizes most strongly to the A_β gene, suggesting that the DC_β cDNA clone represents the human homologue to the mouse A_β gene. Similar numbers of restriction fragments and presumably β genes are present in human DNA (Fig. 5b and C.W. *et al.*, in preparation). These data suggest that there are four to six β genes in the mouse genome. It should be stressed that there may be additional class II genes not sufficiently homologous to be detected by the α and β probes in our hybridization conditions. We can screen for these class II genes as well as for other genes located in the *I* region using the cosmid clones to examine the messenger

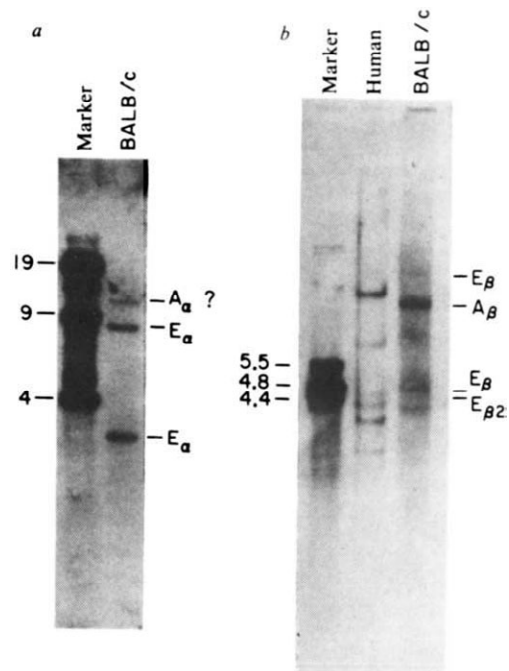
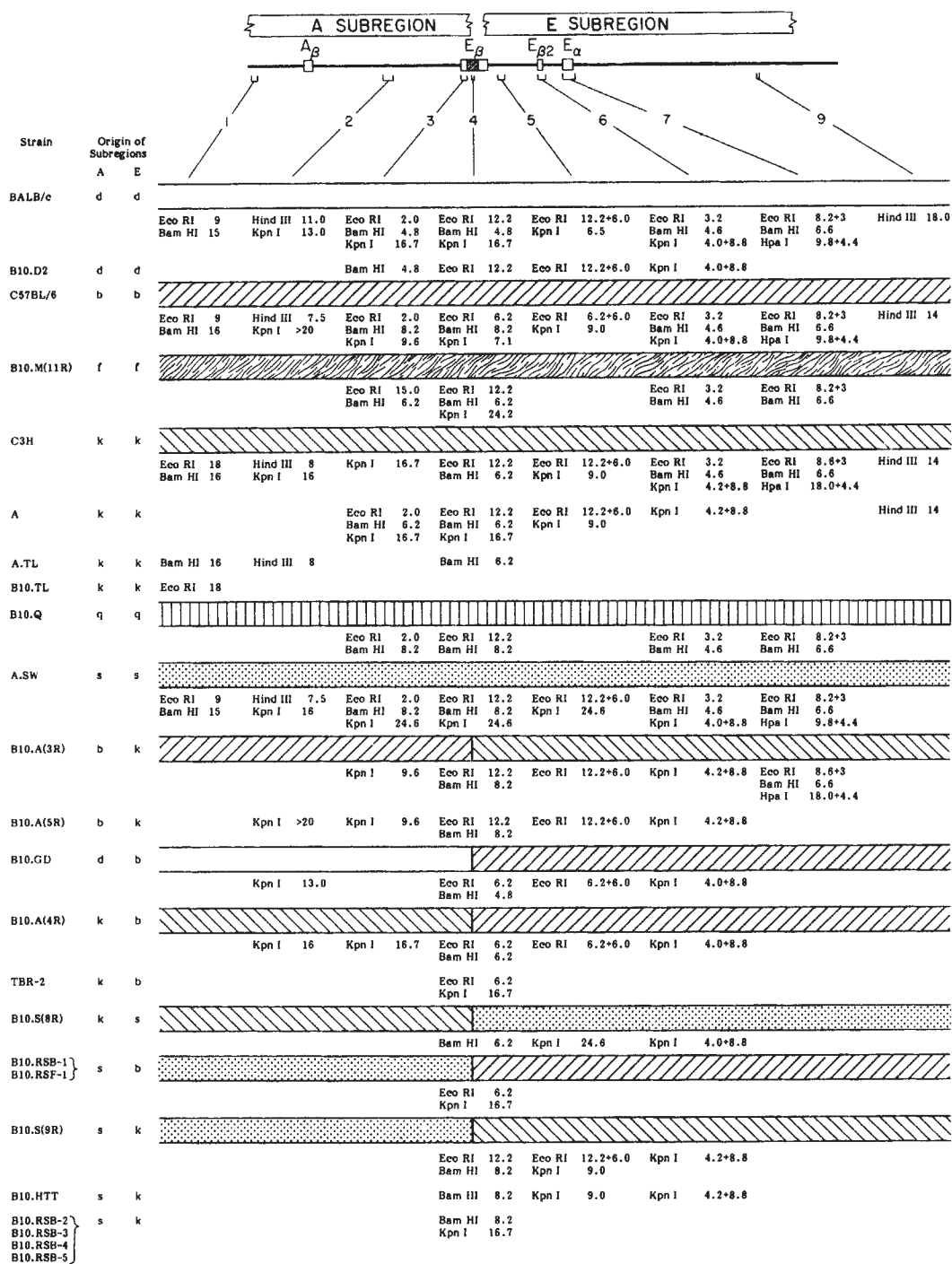


Fig. 5 Southern blot analyses of human and mouse DNAs with the mouse E_α gene and the human DC_β cDNA clone. 5–10 μ g of BALB/c liver and human DNAs were digested to completion with restriction enzyme, separated on a 0.55% agarose gel, transferred to nitrocellulose filter and hybridized using the conditions described in Fig. 3 legend except that mouse DNA was omitted and the final wash was at 65 °C in $2 \times$ SSC, 0.1% SDS. Sizes of marker DNA fragments are given in kilobases. Cloned DNA fragments containing identified class II genes are indicated. *a*, BALB/c DNA was digested with *Eco*RI and hybridized with probe 7 (see Fig. 2). *b*, Human and mouse DNAs were digested with *Bam*HI and hybridized with the insert of the DC_β cDNA clone.



RNAs produced by B cells, T cells and macrophages. Indeed, an additional class II gene exhibiting low homology to the E_{α} gene has been identified between the A_{β} and E_{β} genes with B cell-specific cDNA as a probe (M. Davis, unpublished results).

The E_{α} and E_{β} genes are present in strains not expressing I-E molecules

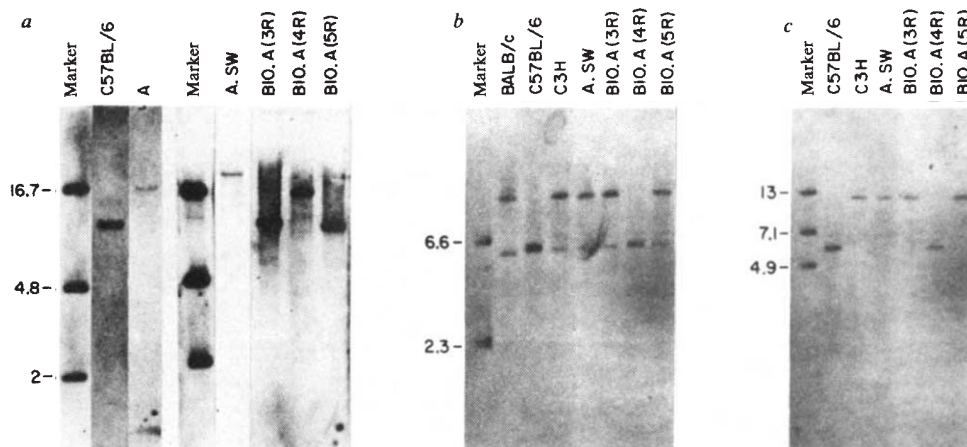
Four inbred strains of mice of independent origin (*b*, *f*, *q*, *s* haplotypes) do not express the I-E molecule on the cell surface³⁸. Two of these haplotypes (*b* and *s*) express the E_{β} polypeptide in the cytoplasm but fail to express the E_{α} polypeptide. Two others (*f* and *q* haplotypes) express neither the E_{β} nor the E_{α} polypeptides. We have used our E_{α} and E_{β} probes in Southern blot analyses to examine the DNAs of these so-called nonexpressor strains for the presence of these genes. Clearly the E_{α} and E_{β} genes are present in all nonexpressor strains (Fig. 6). Accordingly, the failure to express these class II genes

is not a consequence of the deletion of the entire gene. Several different mechanisms might be responsible for the loss of expression at the transcriptional or translational levels.

Correlation of our molecular map with the genetic map of the *I* region

Immunogeneticists have divided the *I* region into five subregions by recombinational analysis of various immune responsiveness traits⁶. The various recombinant congenic strains that we have analysed join in the *I* region two out of four independently derived haplotypes (*d*, *b*, *k* and *s*) which are represented by the inbred mouse strains BALB/c, C57BL/6, A, and A.SW, respectively. The inbred strain A (*a* haplotype) is a natural recombinant between *k* and *d* haplotype genes; since the region of interest to us in this paper in strain A is of *k* haplotype origin we will use *k* instead of *a* when we refer to the haplotype of this strain (see Table 1).

Fig. 7 Southern blot analyses of three recombinant congenic mouse strains and of strains containing their parental haplotypes with probes 3, 4 and 5; 5–10 µg of mouse liver DNAs were digested to completion with restriction enzymes and after gel electrophoresis and transfer hybridized with single-copy probes in the conditions described for Fig. 3 except that the final washes were twice at 65 °C in 0.1×SSC, 0.1% SDS for 15 min each. Sizes of DNA marker fragments are in kilobase pairs. *a*, Digests were done with *KpnI* and the filters were hybridized with probe 3. *b*, *EcoRI* and probe 5. *c*, *EcoRI* and probe 4.



We correlated our molecular map with the genetic map of the *I* region through the analysis of restriction enzyme site polymorphisms as follows. First, 10 single-copy probes from various regions of the 200 kb of cloned DNA were isolated (Fig. 2). By Southern blot analyses of mouse DNA, these probes generally hybridized to single restriction enzyme fragments. Second, these single-copy probes were used to define restriction enzyme site polymorphisms between the four different haplotypes. Third, the polymorphic restriction fragments representing alternative forms of a genetic trait were used to map the probe into an appropriate *I* subregion through the analysis of a panel of recombinant strains. For example, probe 3 representing the 5' half of the E_β gene detected 24.6-, 16.7- and 9.6-kb *KpnI* fragments in the DNAs of A.SW (*s* haplotype), A (*k* haplotype), and C57BL/6 (*b* haplotype) mice, respectively (Fig. 7a). When the three intra-*I* region recombinants B10.A(3R), B10.A(4R) and B10.A(5R) were analysed with probe 3, a 9.6-kb *KpnI* fragment was detected in 3R mice, a 16.7-kb *KpnI* fragment was detected in 4R mice, and a 9.6-kb *KpnI* fragment was detected in 5R mice (Fig. 7a). These data indicate that probe 3 is derived from a region which is of *b*-haplotype origin in 3R and 5R mice but of *k*-haplotype origin in 4R mice. Only the *I*-A subregion or the *K* region could correlate with these restriction site polymorphisms in the three recombinants (Table 1). Since probes 1 and 2 which are located proximal to probe 3 also map to the *I*-A subregion (Fig. 6), we conclude that probe 3, which encodes the 5' half of the E_β gene, maps to the *I*-A subregion. These observations agree with the genetic map.

The surprising finding was that probe 5, which is located ~13 kb distal to probe 3, already maps into the *I*-E subregion although the genetic map places two additional subregions, *I*-B and *I*-J, between *I*-A and *I*-E. Figure 7b shows that probe 5 detects a 12.2-kb (and a 6.0-kb) *EcoRI* fragment in haplotypes *d*, *k* and *s* but a 6.2-kb (and a 6.0-kb) *EcoRI* fragment in the *b* haplotype. This polymorphism between the *b* and the *k* haplotype allows us to map probe 5 by analysis of the recombinants 3R, 4R and 5R unambiguously into the *I*-E subregion (Fig. 7b, Table 1). We further analyse this point below.

The finding that the two most proximal probes 1 and 2 still map into the *I*-A subregion is evident from an analysis of DNAs from B10.TL and A.TL mice and also places the A_β gene in the *I*-A subregion (Fig. 6). The $E_{\beta 2}$ gene (probe 6) and the E_α gene (probe 7) map in the *I*-E subregion (Fig. 6). With probes 8 and 9, isolated from the region distal to the E_α gene, it was more difficult to detect restriction enzyme site polymorphisms. With probe 8 we failed to detect polymorphic restriction fragments. Probe 9, which is located about 60 kb distal to the E_α gene, still maps into the *I*-E subregion (Fig. 6). In at least two recombinant congenic strains, B10.RPD-1 and B10.RPD-2, probe 10, 25 kb distal to probe 9, maps outside the *I*-E subregion, presumably in the *I*-C subregion or the *S* region (not shown). However, in strain B10.F(14R), probe 10 still maps to the *I*-E subregion, indicating that the recombination event in

14R mice has occurred distal to the recombinational events in RPD-1 and RPD-2 mice. These results suggest that the *I*-E subregion is between 90 and 120 kb long.

The *I*-B and *I*-J subregions are confined to a 3.4-kb region of DNA

To determine more precisely the size of the chromosomal region containing the *I*-B and *I*-J subregions, we analysed nine inbred congenic strains with recombination events between the *I*-A and *I*-E subregions. Through Southern blot analyses, we established the restriction maps for the parental haplotypes (*d*, *b*, *k* and *s*) for a region of 27 kb of DNA around the E_β gene using probes 3, 4 and 5 (Fig. 8). Polymorphic restriction enzyme sites which determine by their presence or absence in the appropriate recombinants the rightward and leftward boundaries for the recombination events are circled. The regions between these boundaries which are of undetermined origin in the recombinants, according to our present analysis, are shaded in Fig. 8.

The Southern blotting data for probes 3, 4 and 5 which led to these conclusions are summarized in Fig. 6. Several interesting points can be made from these analyses. All polymorphic restriction enzyme sites which we have analysed around the E_β gene map either to the *I*-A or *I*-E subregions (Fig. 8). No site has been mapped to the *I*-B or *I*-J subregion as defined by the appropriate recombinant strains. According to our present analysis, the maximal distance between the recombination point in B10.GD mice separating the *I*-A from the *I*-B subregion and the recombination point in B10.A(3R) separating the *I*-J from the *I*-E subregion is 3.4 kb. Figure 8 shows that the genome organization around the junction of the *I*-A and *I*-E subregions is very similar in the four mouse strains analysed which are of independent origin. Thus, one cannot postulate that a major deletion or rearrangement affecting the *I*-B and *I*-J subregions has occurred in the BALB/c mouse as compared with parental strains of the *b*, *k* or *s* haplotypes used to generate the recombinants defining the *I*-B and *I*-J subregions.

The *I*-J product may be encoded outside the *I* region

The results described above map the boundaries of the *I*-A and *I*-E subregions into a segment of DNA that is 3.4 kb. This region includes the 3' half of the E_β gene. Indeed, if the E_β genes of the recombinant congenic strains B10.A(3R) and B10.A(5R) are identical, as is suggested by peptide map analysis³⁹, the gap between the *I*-A and the *I*-E subregions may be less than 1 kb. We draw this conclusion because hybridization analysis with the human class II β probe indicates that the E_β gene extends beyond the *Hind*III restriction enzyme site in this region of the BALB/c genome (Fig. 8). The rightward boundary for the recombination event in B10.A(3R) and B10.A(5R) mice is marked by the polymorphic *EcoRI* restriction site 1 kb distal to this *Hind*III site (see Fig. 8).

This does not leave much DNA to encode the traits of the *I-B* and *I-J* regions. Indeed, the *I-B* subregion has only been defined based on the control of certain immune responses⁶. Recently, a controversy has arisen as to whether the *I-B* subregion exists in that the immune responses it controls may actually be regulated by both the *I-A* and *I-E* class II molecules⁴⁰. Cloning studies and DNA sequence analyses of the recombinant regions defining the *I-B* subregion should allow us to localize this subregion even more precisely and, indeed, to determine whether it exists.

The *I-J* subregion apparently controls the expression of two or more serologically defined *I-J* gene products⁴¹⁻⁴³. Several models may explain the gene or genes encoding *I-J* product(s). First, the *I-J* gene may be encoded outside the *I* region. This possibility cannot be excluded because the generation of the recombinant congenic strains used to define the *I-J* subregion also involved recombination events outside the *I* region on chromosome 17. However, this possibility seems unlikely because *I-J* appears to segregate with the *I* region and not with any other region of the *H-2* complex. Second, expression of the *I-J* gene product may be controlled by a regulatory element which is encoded in this 3.4-kb region. Again this possibility seems unlikely because of the polymorphism observed in congenic mice with identical background genes (that is, non-*I*-region genes). Third, *I-J* polypeptides might have been selected during ontogeny from a pool of variants for their ability to bind the E_β polypeptide in interactions between suppressor T cells and B cells or macrophages. Mouse strains polymorphic for E_β as well as recombinants affecting the E_β gene would thus select for different *I-J* polypeptides. The *I-J* polypeptide therefore seems to be under control of a locus overlapping the E_β gene, although its structural gene(s) would map elsewhere. Fourth, the *I-J* product may arise from RNA splicing patterns involving a polymorphic exon encoded between the *I-A* and *I-E* subregions and additional, nonpolymorphic exons encoded in the *I-A* or *I-E* subregions. This model could be analogous to the alternative RNA splicing patterns seen for the secreted and membrane forms of immunoglobulin heavy chain genes. Finally, the *I-J* and E_β polypeptides may be identical. The *I-J* chain, however, may differ in its antigenic properties from the E_β chain because of post-translational modifications such as glycosylation, or because of conformational differences if it is associated with a polypeptide different from E_α or alternatively, if it occurs as a single polypeptide chain on the surface of T suppressor cells.

Studies in progress should allow us to distinguish the first three from the latter possibilities by using a subcloned DNA segment between the *I-A* and *I-E* subregions to carry out RNA blot analyses on messenger RNAs from T-cell hybridomas expressing *I-J* gene products. The last two hypotheses would suggest that this region should cross-hybridize to *I-J* messenger RNAs, whereas the former three possibilities argue that the structural gene for the *I-J* polypeptide lies outside the *I* region and, accordingly, no cross-hybridization should be observed. We will also determine by cloning and sequencing, the exact location of the recombination points in, for example, B10.A(3R) and B10.A(5R) mice which define the leftward and rightward borders of the *I-J* subregion. Indeed, it is possible that the *I-A* and *I-E* subregions will be contiguous with no intervening DNA that could encode the *I-B* or *I-J* subregions. If so, the *I-B* and *I-J* subregions cannot be located between the *I-A* and *I-E* subregions.

Recombination in the *I* region is sharply localized in and around the E_β gene

The recombination events which occurred in nine independently generated recombinant congenic mice between the *I-A* and *I-E* subregions have been mapped (Fig. 8). These recombination points all map near to the E_β gene within a 9.8-kb segment of DNA. Indeed, serological and peptide map analyses suggest that the recombinational event in B10.GD mice oc-

curred in the E_β gene^{34,36}. This result is in agreement with our chromosomal mapping studies using restriction enzyme site polymorphisms. We carried out several types of control experiments. First, in certain cases we analysed doubly recombinant strains of mice which had been generated by genetic crosses between one of the above recombinant congenics and a new parental strain. In each case where the *I*-region recombinant haplotype was retained, the same polymorphic restriction fragments were observed as in the original parental strains (Table 1, Fig. 6). Second, critical strains B10.A(3R), B10.A(5R), B10.S(8R), B10.S(9R) and B10.HTT were obtained from other laboratories (D. Murphy and C. David) and gave the same results as the identical strains raised in our colony (J.F.). From a comparison of the lengths of those restriction fragments (Fig. 6) which span the recombination points in the appropriate recombinants with the maps of the parental haplotypes in Fig. 8, it is clear that the recombination events have occurred without any major duplication or deletion of DNA sequences. For example, in Fig. 7c, a 12.2-kb *EcoRI* fragment is detected in B10.A(3R) and B10.A(5R) and a 6.2-kb fragment is found in B10.A(4R) with probe 4 which is derived from the large intervening sequence in the E_β gene. As can be seen from Fig. 8, these *EcoRI* fragments span the recombination points in the recombinants. The sizes of these *EcoRI* fragments support the

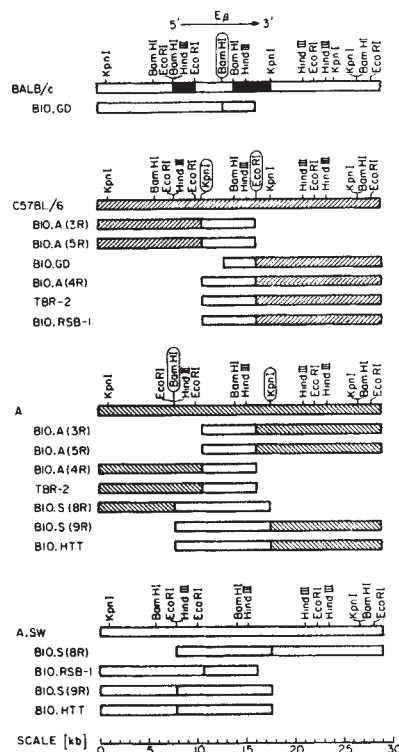


Fig. 8 Partial restriction maps for 27 kb of DNA around the E_β gene in mouse strains of *d*, *b*, *k* and *s* haplotypes. These maps indicate the regions where crossing-over occurred in nine independently generated recombinant congenic strains. The restriction maps for the four mouse strains were obtained by Southern blot analyses of single and double digestions of mouse liver DNAs with the indicated enzymes using probes 3, 4 and 5 as hybridization probes (see Fig. 2 for the location of these probes). Compared with Fig. 2, the map for BALB/c shows only the restriction sites mapped by Southern blotting. Maps obtained by Southern blotting might not reveal all sites for the enzymes used since sites outside the fragments detected with the hybridization probes are not seen. This limitation of the method, however, does not affect any of our present conclusions. The location and maximal size of the 5' and 3' exons of the E_β gene are indicated in the BALB/c map by black boxes (see Fig. 2 legend for a discussion of the organization of this gene). Those regions of the four chromosomal DNA segments that are present in the nine recombinant congenic mouse strains according to our analyses, are indicated by appropriately marked bars below the restriction maps. The grey boxes indicate the regions in the recombinants where the crossing-over has occurred and which cannot be assigned at present to either parent because of the lack of polymorphic restriction sites. Polymorphic restriction sites confining the regions of recombination are circled.

hypothesis that the recombinants have been generated by a homologous crossing-over event.

The recombinational events in the nine independently generated recombinants analysed could all have occurred at precisely the same site. As discussed above, we are in the process of subcloning several of these recombinant regions to identify in molecular terms their precise points of recombination. These observations suggest that in the *I* region, recombinational events are not random but rather occur in certain highly localized regions. This obviously has important implications for any attempts to correlate genetic maps that are determined by recombinational analyses and molecular maps determined by direct cloning. For example, the A_β and the E_α genes are by recombinational analyses about 0.1 centimorgan apart². As the mouse genome is 1,600 centimorgans long and contains about 3×10^6 kilobase pairs, 0.1 centimorgan corresponds to about 200 kb (if on the average recombination is random throughout the mouse genome). We have now established directly that the A_β and E_α genes are separated by about 85 kb of DNA (Fig. 2) and that all the recombinational events analysed in this region occurred within 9.8 kb of DNA. One wonders if genetic maps for other regions of the eukaryotic genome will show similar recombinational preferences. In particular, it will be interesting to determine whether there are other hot-spots of recombination within the major histocompatibility complex, as for example between the *I-E* subregion and *S* region or between the *S* and *D* regions—two sites where many cross-overs have been mapped.

Received 2 August; accepted 23 September 1982.

1. Klein, J. *Biology of the Mouse Histocompatibility Complex* (Springer, Berlin, 1975).
2. Snell, G. D., Dausset, J. & Nathanson, S. *Histocompatibility* (Academic, New York, 1976).
3. Nagy, Z. A. *et al. Immun. Rev.* **60**, 59–83 (1981).
4. Klein, J. *et al. Nature* **291**, 455–460 (1981).
5. Benaceraff, B. *J. Immun.* **120**, 1809–1812 (1978).
6. Murphy, D. B. in *The Role of the Major Histocompatibility Complex in Immunology* (ed. Dorf, M. E.) 1–32 (Garland STPM, New York, 1980).
7. Krupen, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1254–1258 (1982).
8. Wieder, K. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3599–3603 (1982).
9. Taniguchi, M. *et al. Nature* **298**, 172–174 (1982).
10. Klein, J. & Figueroa, F. *Immun. Rev.* **60**, 23–57 (1981).
11. Steinmetz, M. *et al. Cell* **24**, 125–134 (1981).
12. Steinmetz, M. *et al. Cell* **25**, 683–692 (1981).
13. Moore, K. W. *et al. Science* **215**, 679–682 (1982).
14. Steinmetz, M. *et al. Cell* **28**, 489–498 (1982).
15. Goodenow, R. S. *et al. Science* **215**, 677–679 (1982).
16. Woodward, J. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3613–3617 (1982).
17. Örn, A. *et al. Nature* **297**, 415–417 (1982).
18. Wake, C. T. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
19. Long, E. O. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
20. McMillan, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 5135–5139 (1977).
21. Allison, J. P. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3953–3956 (1978).
22. Shackelford, B. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 4566–4570 (1981).
23. Goyert, S. M. *et al. J. exp. Med.* **156**, 550–566 (1982).

The future

The cloning of the *I* region will permit the precise identification of genes involved in immune responsiveness and other phenotypic traits encoded by this region. We will be able to use the techniques of gene transfer and *in vitro* mutagenesis to determine which genes control specific traits and how they function to regulate these traits. The cosmid clones encompassing the *I* region can be used to determine whether other previously undetected *I*-region genes are expressed in cells that control various aspects of the immune response such as B cells, T cells or macrophages. The detailed knowledge of the *I-A* and *I-E* loci will be of great value in the analysis of the human *I* region at the molecular level. Finally, it is clear that the genetic map of the *I* region can now start to be replaced with a molecular map which localizes precisely the genes encoding the various types of class II polypeptides.

We thank Elizabeth Gibb for technical assistance, Joan Kobori for a gift of probe 3, Bernita Larsh for the preparation of the manuscript, and all members of the Hood group for stimulating discussions. We also thank P. Singer and J. Klein, D. Murphy and C. David for various congenic mouse strains. M.S. was supported by the Deutsche Forschungsgemeinschaft and by a Senior Lievre Fellowship from the California Division of the American Cancer Society (S-48-82). J.M. is a postdoctoral fellow of the Arthritis Foundation. J.F. is the recipient of an American Cancer Society Faculty Research Award. The work was supported by grants from the NIH.

24. Shaw, S. *et al. J. exp. Med.* **156**, 731–743 (1982).
25. Bono, R. & Strominger, J. L. *Nature* **299**, 836–838 (1982).
26. Lee, J. S., Trowsdale, J. & Bodmer, W. F. *Proc. natn. Acad. Sci. U.S.A.* **79**, 545–549 (1982).
27. Wiman, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1703–1707 (1982).
28. Korman, A. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1844–1848 (1982).
29. Larhammar, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3687–3691 (1982).
30. Larhammar, D. *et al. Cell* **40**, 153–161 (1982).
31. Kratzin, H. *et al. Hoppe-Seyler's Z. physiol. Chem.* **362**, 1665–1669 (1981).
32. Pierres, M. *et al. J. Immun.* **126**, 2424–2429 (1981).
33. McNicholas, J. *et al. Science* (in the press).
34. Jones, P. P. *J. exp. Med.* **152**, 1453–1458 (1980).
35. Rose, S. M. & Cullen, S. E. *J. Immun.* **127**, 1472–1477 (1981).
36. Plunkett, M. L. *et al. J. exp. Med.* **155**, 937–942 (1982).
37. Kaufman, J. F. & Strominger, J. L. *Nature* **297**, 694–697 (1982).
38. Jones, P. P., Murphy, D. B. & McDevitt, H. O. *Immunogenetics* **12**, 312–327 (1981).
39. Cook, R. G. *et al. J. exp. Med.* **149**, 981–986 (1979).
40. Baxevanis, C. N., Nagy, Z. A. & Klein, J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3809–3813 (1981).
41. Murphy, D. B. *Springer Semin. Immunopath.* **1**, 111–130 (1978).
42. Niederhuber, J. E. *et al. J. Immun.* **122**, 1342–1349 (1979).
43. Ochi, A. *et al. J. Immun.* **129**, 227–231 (1982).
44. Dretzen, G. *et al. Analyt. Biochem.* **112**, 295–298 (1981).
45. Steinmetz, M. *et al. Nucleic Acids Res.* **8**, 1721–1729 (1980).
46. Beacage, S. L. & Caruthers, M. H. *Tetrahedron Lett.* **22**, 1859–1862 (1981).
47. Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).

LETTERS TO NATURE

Convective cloud merging and its effect on rainfall

D. A. Bennetts, M. J. Bader & R. H. Marles

Meteorological Office, Bracknell, Berkshire RG12 2SZ, UK

An important aspect in the study of cumulonimbus convection is the identification of situations that lead to intense rainfall. We examine here one such situation in which two initially separate clouds interact and merge. The process is found to depend on the variation of wind with height, the relative stage of development of the two clouds and their initial separation. A numerical simulation is described in which merging is shown to increase the total surface precipitation by a factor of four.

Simpson *et al.*¹ investigated the development of merging cumulus when the two separate clouds were initially two or three cloud diameters apart. They suggested that new growth could take place above colliding gust fronts formed by the

downraughts from each cloud. Large accumulations of rainfall resulted from the increased size and duration of the cloud complex. Here an alternative mechanism of merging is considered in which the amount of rain is increased by enhancing the rainfall rate². The work is a development of an idea discussed by Orville *et al.*³ in which two separate cumulonimbus clouds grow in close proximity, closer than that necessary for the merging process described by Simpson *et al.* Under favourable combinations of vertical wind shear, cell separation and relative cloud development, the cloud motion fields (as opposed to the downraughts) interact and the clouds merge to form a single entity.

The effects of this type of merging should be evident in the rainfall characteristics of a field of convective clouds, particularly if those clouds are similar in size and growing in uniform conditions. On such occasions most clouds might be expected to have similar rainfall, but a few, growing sufficiently close, would merge, the consequent enhancement in rainfall rate being dependent on the effectiveness of the merging. Such rainfall characteristics have been observed using a weather radar^{4,5}. The peak rainfall rate of most of the clouds fell within

Table 1 A comparison of the water budgets of the single cell and merged clouds

	Single cell	Merger	Difference merger – single
1 Total available condensed cloud substance CN	3.8×10^8 kg	10.12×10^8 kg (Fractions per unit CN)	+33%
2 Total precipitation generated within the cloud comprising of	0.55	0.61	+0.064 (+12%)
a, accretion of cloud water by rain and hail	0.23	0.31	+0.080 (+35%)
b, other processes (such as autoconversion of cloud water to rain, sublimation of cloud ice to graupel)	0.32	0.30	-0.016 (-5%)
3 Precipitation evaporated below cloud base	0.47	0.39	-0.086 (-18%)
4 Net precipitation reaching the ground as rainfall	0.07	0.22	+0.15

Water budgets: 1, The net condensed cloud substance (CN): the percentage change in the third column is calculated using CN from two single cells. 2, The total amount of precipitation produced within the cloud together with a breakdown of the physical processes involved. 3, The evaporation. 4, The surface rainfall, that is the total amount produced minus that evaporated. All values in 2, 3 and 4 are expressed as fractions of CN.

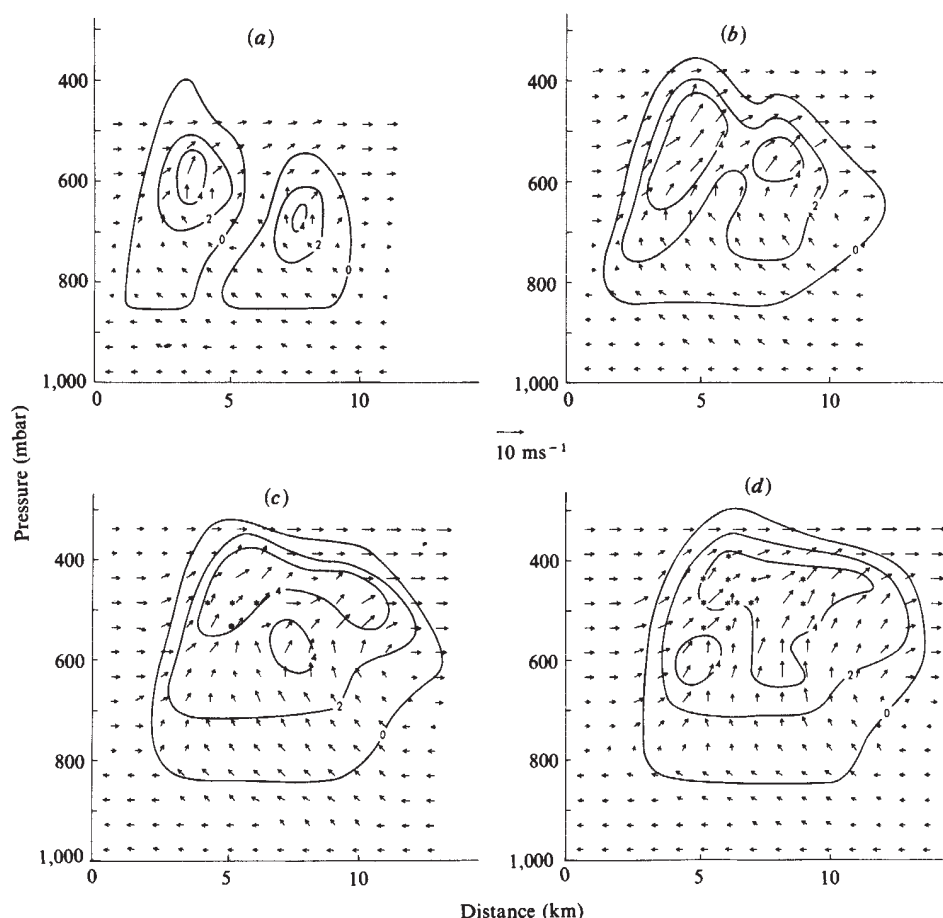
the range $5\text{--}15\text{ mm h}^{-1}$, but up to 30% had a much higher rainfall rate, in the range $30\text{--}100\text{ mm h}^{-1}$. It has been suggested that the second group might have resulted from merging. To investigate this, both single and merging clouds have been simulated with a tuned three-dimensional numerical model⁶ using representative thermodynamic and dynamic data⁵. Details of the microphysical and turbulent parametrization schemes, together with the model boundary conditions, are given in ref. 6, but for brevity are omitted here.

Figure 1 shows the early development of two clouds, separated in distance by 7 km and in time of initiation by 6 min. Each shows a vertical section through the centre of the cloud parallel to the direction of the mean wind. At a simulated cloud time of 12 min the motion field of the larger cloud has not developed sufficiently to influence the growth of the other and both clouds lean to the right, as would be expected in the prevailing conditions. By 16 min the clouds have merged perceptibly. Large ice particles begin to form in the more mature cloud and, by 20 min, their concentration has reached 0.05 g per kg and their fall speed, in still air, is $\sim 6\text{ m s}^{-1}$ (ref. 6). At

this stage they can be considered as 'embryo precipitation particles'.

The instantaneous velocity vectors are evaluated relative to the mean speed of the larger cloud which corresponds to the wind at 725 mbar ($\sim 3\text{ km}$ above the surface) in the undisturbed air. Relative to this frame of reference the inflow and outflow are clearly evident. The clouds move at slightly different speeds because of their different size (which affects their interaction with the undisturbed flow), and their mutual interaction. As a result, the region of high liquid water content ($>2\text{ g per kg}$) in the lower part of the right hand cloud is advected towards the left (relative to the frame of reference defined above), and by 24 min (Fig. 1d), is positioned so that subsequently the precipitation falls through it, the growth of the precipitation thereby being greatly enhanced by accretion of numerous small water droplets. This process, whereby precipitation generated in one cloud is augmented as it passes through another, is similar to that encountered near high ground⁷ where precipitation generated from frontal cloud grows by accretion of orographically induced cloud which forms lower down, just above the hill.

Fig. 1 Distribution of cloud content (water and ice) in g per kg within the clouds at a simulated time of: a, 12 min; b, 16 min; c, 20 min; d, 24 min. Arrows denote wind velocities in the plane of the diagram relative to the mean speed of the left hand cloud. The presence of rain (•) and hail (*) is also indicated.



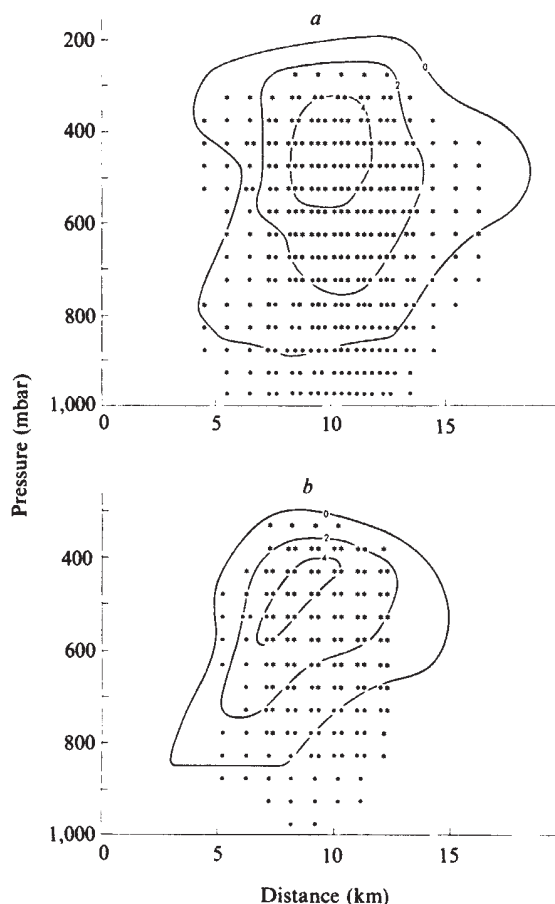


Fig. 2 Distribution of rain (•), hail (*) and cloud substance (water and ice) at a simulated time of 36 min within: *a*, the merged cloud; *b*, the single cell. Isopleths of cloud content are in g per kg. The number of symbols in each group represents concentrations of <0.5, 0.5–2.0 and >2.0 g per kg. These approximate to rainfall rates of <8, 8–44 and >44 mm h⁻¹ respectively.

The second region of high liquid water content low down on the left hand side of the cloud in Fig. 1*d* cannot influence the growth of the precipitation by accretion because it is in an unfavourable position. During the subsequent development it rises to the top of the cloud to provide a continuing supply of precipitation embryos. A later stage in the development when merging is complete is shown in Fig. 2*a*. There is a high concentration of rain and hail in the centre of the cloud and the maximum modelled instantaneous rainfall rate at the surface (1,000 mbar) is ~100 mm h⁻¹. Comparison between this and a modelled single cell cloud (Fig. 2*b*) growing in the same environment shows that the merged cloud produces ~4 times more total surface precipitation than two individual clouds growing separately, and there is a 10-fold increase in the maximum instantaneous surface rainfall rate. (Both the merged and single clouds have a similar size and structure in the cross-shear plane.)

The rainfall characteristics of both simulated clouds are shown in Fig. 3 together with observations⁵ from radar. The observed rainfall rates, averaged over 4 km², are the highest within the radar echo of the cloud; the modelled rates are the means of the four grid values (each 1 km²) centred on the simulated peak surface rainfall. Both sets of values are subject to some uncertainty. Calibrated radar observations are generally considered to be representative to within a factor of ~1.5 but it is difficult to be as precise in estimating the errors in the numerical model. There are uncertainties in the parametric representation of the microphysical and turbulent diffusion processes as well as in the effects of the lateral boundary conditions. An attempt has been made to 'tune' the model⁶ to reduce these uncertainties but it would be unwise to suggest

that the predictions had an accuracy greater than a factor of 2, although a direct comparison between simulations has the merit that many of the uncertainties at least remain constant. In consequence, the agreement between observed and modelled peak rates is fortuitously close, but there is no doubt about the difference in rainfall rates between the single cell and merger clouds.

Comparison between the two simulations shows that more rain reaches the ground from the merger because more precipitation is produced by accretion within the cloud and there is less evaporation below cloud base. The relevant quantities are shown in Table 1. The production of precipitation by accretion and loss by evaporation are expressed as fractions of the mass of cloud substance (water and ice) available for the production of precipitation. This quantity (CN) represents the difference between the cloud substance condensed and that evaporated at the cloud boundary.

The total precipitation produced per unit CN within the merged cloud is 12% greater than that within the single cell because a higher proportion of cloud water is accreted in a merger. The proportion of precipitation generated by other microphysical processes is similar in both clouds; in fact, there is slightly less in a merger because the cloud water content has been depleted by accretion. In addition, the amount of precipitation lost by evaporation below a merger is 18% less than that below a single cell. This results in the fraction of precipitation reaching the ground from the merger being three times greater than that from the single cell. However, CN in the merger is more than twice that in the single cell and, expressed in absolute terms, the mass of precipitation reaching the ground from the merged cloud is four times that from two single cells.

The increased efficiency of precipitation production within the merger can partly be explained by its increased size. The relevant parameter is the horizontal displacement of a precipitation particle as it falls compared with the 'width' of the cloud. This determines the proportion of time spent by the particle accreting water inside the cloud to that spent evaporating outside it. The efficiency also depends on the dynamic interaction of the clouds which determines the relative position of the precipitation and regions of high liquid water content.

At present the relative importance of cloud size and the dynamical effects of merging are not known. However, supple-

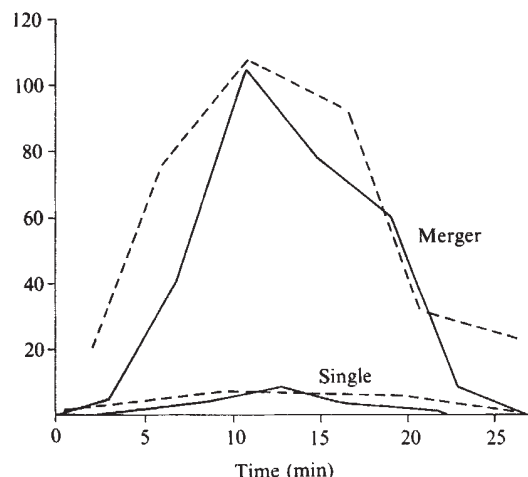


Fig. 3 Simulated and observed rainfall rates for merged and single cell clouds. The dashed curve labelled 'single' depicts observed rainfall rates that were typical of the smaller clouds and the dashed curve labelled 'merger' the rainfall rates of the largest cloud observed⁵. The largest was chosen as the numerical simulation (solid line) shows the results of the most efficient merger achieved. Note that, in the text, comparisons are between mergers and two single cell clouds. The simulation shown here labelled 'single' (solid line) represents one single cell cloud to enable direct comparison with observations.

mentary numerical simulations have shown that merging can both increase the surface rainfall by a factor of four, and decrease it by $\sim 40\%$. A reduction in rainfall can occur when the wind field of the primary cloud dominates that of the secondary which, in the limit, is drawn into the updraught of the primary cloud before it has grown past its embryonic stage. Consequently the large variability often observed in convective precipitation⁵ can be accounted for even without considering the natural environmental variability that is almost certainly present within any air mass.

Received 28 June; accepted 22 September 1982.

1. Simpson, J., Westcott, N. E., Clerman, R. J. & Pielke, R. A. *Arch. Met. Geophys. Bioklim.* **A29**, 1–40 (1980).
2. Barrett, E. C. *Proc. Int. Training Course*, Rome, 97–103 (1980).
3. Orville, H. D., Kuo, Y.-H., Farley, R. D. & Hwang, C. S. *J. Rech. atmos.* **14**, 499–516 (1980).
4. Browning, K. A. *Met. Mag.* **108**, 161–184 (1979).
5. Bennetts, D. A. & Bader, M. J. in *Cloud Dynamics* (Reidel, Dordrecht, in the press).
6. Bennetts, D. A. & Rawlins, F. Q. *Jl R. met. Soc.* **107**, 477–502 (1981).
7. Hill, F. F., Browning, K. A. & Bader, M. J. *Q. Jl R. met. Soc.* **107**, 643–670 (1981).

Cyclic thermal transformations in caesium graphite

A. R. Ubbelohde & I. Drummond

Imperial College, Department of Chemical Engineering and Chemical Technology, London SW7 2BY, UK

Various physical properties can yield novel information about effects of texture of a graphite, on the detailed course of any thermal transformation of a graphite intercalate, when it is subjected to cyclic changes of temperature including a lambda peak. Using a DSC calorimeter with an imposed temperature change of 10 K min^{-1} , we have found a novel transformation in caesium graphite CsC_8 around 250 K. We show here that fine details appear to be sensitive to the texture of the graphite used.

When electron donor or electron acceptor molecules intercalate into graphite, this pushes the carbon hexagon sheets apart by several nanometres. Any occasional defect-bonding between neighbouring parallel carbon hexagon sheets becomes stressed by this enforced separation, and in near-ideal ordered graphites 'rogue bonds' between the sheets may even snap. On lessening this stress by removing the intercalated molecules, in favourable cases the rogue bonds may not reform. Planned sequences of intercalation and removal might thus in principle be made a means for permanently improving the quality of well ordered graphites. Unfortunately, the graphites and pyrographites available in the early experiments were not sufficiently well ordered to test sequences of intercalation and removal adequately. It was found that a proportion of the intercalated molecules remained 'locked-in', as so called 'residue compounds'¹. Use of better oriented graphites, as prepared by subjecting pyrographites to stress annealing^{2–4}, gives a less confused response to sequences of intercalation and removal of the intercalated molecules, particularly when optimum behaviour of the lamellar framework is attained, by careful quality selection⁵ of highly oriented pyrolytic graphite (HOPG).

When a graphite intercalate undergoes one or more thermal transformations on traversing a range of temperatures, even more gentle stresses can be imposed through cyclic changes of temperature; for example, there are changes of structure but no changes of composition in cycling the temperature around a lambda point. With this in view, various thermal transformations in graphite intercalates are being studied. These present some novel features in the chemical physics of solids, where previous research has been largely restricted to three-dimensional structures. Quite often a choice can be made between several different physical properties for study in relation to the thermal transformation. For example, the lambda

transformation in graphite nitrates and deuterates with a peak temperature around 252 K has been followed in cyclic temperature changes using observations of changes in thermal expansion, lattice structure, electrical resistivity, thermal e.m.f., and pulsed NMR^{6–9}. Because of the extremely high anisotropy of these compounds, as the temperature is varied the sharpness of peak change in any one layer may differ notably from the (often much lessened) sharpness of the correlation between neighbouring layers. In the limit, each layer of intercalate might be effectively independent of even the nearest neighbour layers; effectively two-dimensional thermal transformations would be of considerable theoretical importance as well as practical interest. But in practice, at least weak correlation of molecular behaviour can generally be observed between neighbouring layers, up to (say) five away. These considerations make it desirable whenever feasible to choose physical properties which can be conveniently studied parallel and perpendicular to the layers, independently. For example, to enlarge the possibilities, methods of measuring resistivity changes perpendicular to the layers have recently been improved⁵.

Knowledge of the enthalpy and entropy changes provides important background information for interpreting any of the other physical measurements on transformations in intercalation compounds. A 'rate of enthalpy change' calorimeter has recently become available which has the advantage of requiring only a few milligrammes of substance. Despite certain limitations, such as the rather fast changes of temperature that must be used, this calorimeter has given interesting preliminary information about graphite nitrates¹⁰. It has now been used with caesium graphites. Both the first stage and second stage intercalates have been studied, but for simplicity the present report deals principally with the first stage intercalate CsC_8 . Unexpectedly, calorimetry reveals a thermal transformation in the temperature range around 250 K, which has not been hitherto detected either in resistivity studies, or detailed X-ray diffraction measurements⁹ in CsC_8 .

Our general methods followed regular two-bulb preparative techniques. To permit comparisons between graphites of different textures each preparation exposed four different specimens of $\sim 25\text{ mg}$ side by side to the caesium vapour, in conditions appropriate for obtaining the first or the second stage intercalate. After completion of intercalation the specimens were covered with non-reactive petroleum jelly¹¹. It was verified that *per se* this film of jelly (which facilitates handling particularly after intercalation) makes a negligible contribution to the calorimetric anomalies now reported: interestingly the jelly showed a very weak glass-type transformation at considerably

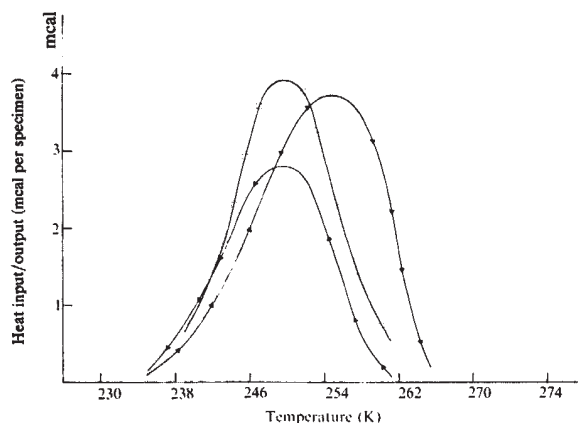


Fig. 1 Heat input/output for two different specimens of CsC_8 . For HOPG ($\blacktriangledown$) the direction of temperature changes imposed follows the arrows. For Ticonderoga (natural) graphite ($\square$) only the cooling curve is shown.

higher temperatures, but this could not be shown on the scale used for Fig. 1.

Following earlier indications of its importance (see ref. 12) in the present studies particular attention was paid to the textural quality of the graphites used for intercalation. Two graphites of different origins were used. Figure 1 refers to stress-annealed pyrographite carefully selected by us from samples of HOPG (provided by A. Moore of Union Carbide), and to a piece of Ticonderoga graphite broken off from a fairly large specimen of this geological deposit. As anticipated, the thermal behaviour of CsC_8 although prepared under identical vapour pressure of caesium differed in fine detail, depending on which graphite was intercalated, and whether the temperature was rising or falling through the transformation, roughly between 238 and 262 K (Fig. 1). The origin of rate dependent differences between the two samples cannot yet be pinned down with certainty. Rate-controlled input or abstraction of heat is used with the present type of calorimeter; so far, only heat transfers accompanying temperature changes of 10 K min^{-1} have been used exhaustively. With such a simple type of intercalate as atoms of caesium, many solid state processes should be too rapid to be distorted by the rates of change of temperature imposed on the calorimeter. Provisionally, the findings suggest some kind of order/disorder, with details controlled by the texture of the carbon-hexagon layers in the two varieties of graphite studied; suggestions have been made with

reference to transformations in CsC_{24} (ref. 9) which appear to be related to the thermal transformations now reported in CsC_8 .

The magnitude of the thermal transformation is also noteworthy. Approximate integration of the calorimetric data indicates a total enthalpy change of about 26.7 cal per g atom, with an entropy change of only about 0.1 e.u.

Observations that the texture of the graphite can have such marked influence on the fine detail of thermal transformations of intercalates emphasize the need for graphites of better quality than any yet available, when seeking to establish unusual two-dimensional effects such as phonon-electron interactions and superconducting transitions.

Note that although the choice of intercalates with such strong electron donor character as caesium for the present research has been made because they present a special theoretical interest, they also enhance the practical difficulty that these intercalate atoms are highly reactive chemically. Our experience with this novel calorimeter is limited. Despite all the precautions taken against the intrusion of reactive species other than graphite, and caesium, part of the interesting non-reversibility which may be seen in Fig. 1, could be due to some permanent loss of caesium in each cycle, by combination with other intruder molecules. However, a dominant role of the texture of the graphite, in determining fine differences between different specimens, seems certain.

Received 17 August; accepted 15 September 1982.

1. Ubbelohde, A. R. & Lewis, F. A. *Graphite and its Crystal Compounds* (Oxford University Press, 1960).
2. Moore, A. W., Ubbelohde, A. R. & Young, D. A. *Proc. R. Soc. A* **280**, 153–155 (1964).
3. Ubbelohde, A. R. *Carbon* **14**, 1–5 (1976).
4. Tzuzuku et al. *J. phys. Soc. Jap.* **51**, 1476–1481 (1982).

5. Drummond, I. & Ubbelohde, A. R. *Proc. R. Soc. A* **371**, 309–318 (1980).
6. Bottomley, M. J., Parry, G. S. & Ubbelohde, A. R. *Proc. R. Soc. A* **279**, 291–301 (1964).
7. Nixon, D. E., Parry, G. S. & Ubbelohde, A. R. *Proc. R. Soc. A* **291**, 324–339 (1966).
8. Ubbelohde, A. R. *Proc. R. Soc. A* **304**, 25–43 (1968).
9. Onn, D. G., Foley, G. M. T. & Fischer, J. E. *Mater. Sci. Engng* **31**, 271–275 (1977).
10. Dworkin, A. & Ubbelohde, A. R. *Carbon* **16**, 291 (1979).
11. Murray, J. J. & Ubbelohde, A. R. *Proc. R. Soc. A* **312**, 371–380 (1969).
12. Ubbelohde, A. R. *Carbon* **7**, 523–530 (1969).

Pu, ^{241}Am and ^{137}Cs in soil in West Cumbria and a maritime effect

R. S. Cambray & J. D. Eakins

Environmental and Medical Science Division, AERE Harwell, Oxon OX11 0RA, UK

Discharges to the Irish Sea from Sellafield (formerly Windscale) in Cumbria in the period 1957–79, included about 16,000 Ci of Pu α activity and 800,000 Ci of ^{137}Cs , in addition to other actinides and fission products^{1–8}. Most of the ^{137}Cs in the sea remains in solution whereas ~95 % of the Pu is removed to sediments⁷. To ascertain whether any of this discharged material was being transferred back to land, samples of soil were taken along two transects normal to the Cumbrian coast and extending up to 20 km inland. We report here that Pu and Am deposits decrease with distance inland and correlate with deposits of marine-derived sodium. An enrichment of actinides in seaspray relative to seawater is required to account for the observed deposits.

Samples from the first transect, between St Bees and Ennerdale, were collected in May 1977 and from the second, between Nethertown and Newlands, in July 1979. The locations of these transects and the individual sampling sites are shown in Fig. 1. Soil was taken from apparently undisturbed sites to a depth of 15 cm using core tubes of 3.8-cm diameter. Three cores were taken at each location, combined, dried at 105 °C, ground to a particle size of <150 μm and homogenized. Approximately 100 g of each bulked sample were taken for analysis by γ -ray spectrometry, using lithium drifted germanium detectors. ^{137}Cs was determined from the spectra by least squares fitting using automatic data processing^{9,10}. Plutonium was determined on 70 g samples by an acid leaching technique¹¹, using ^{242}Pu as an internal tracer. The amounts of ^{238}Pu and $^{239+240}\text{Pu}$ in electrodeposited sources were determined by α spectrometry. Soils

from the Nethertown transect were also analysed for ^{241}Am by radiochemistry and α spectrometry, using ^{243}Am as a tracer. The analytical results are presented in Table 1, radionuclide concentrations being expressed as deposits per cm^2 to a depth of 15 cm.

To evaluate the results, the deposit of radionuclides in soil due to fallout from distant nuclear weapons must be considered. The accumulated deposit depends mainly on rainfall¹² and latitude¹³. Results from a nationwide survey¹⁴ have shown that, in Britain, the ^{137}Cs deposit (to a depth of 15 cm) is ~7.5 pCi cm^{-2} per m of rain. The $^{239+240}\text{Pu}$ deposit is ~0.14 pCi cm^{-2} and that of ^{238}Pu , including a contribution from a satellite burnup¹⁵ in 1964, is ~0.006 pCi cm^{-2} . The fallout baseline for ^{241}Am is not so well established. However, the mean ratio of ^{241}Am to $^{239+240}\text{Pu}$ on the Nethertown transect between 10.9 and 20.5 km inland is 0.23, a value which agrees with the global estimate of Krey et al.¹⁶. This ratio suggests a ^{241}Am deposit from the testing of nuclear weapons of ~0.032 pCi cm^{-2} per m of rain. The weapons fallout contribution at each site has been estimated from the mean annual rainfall

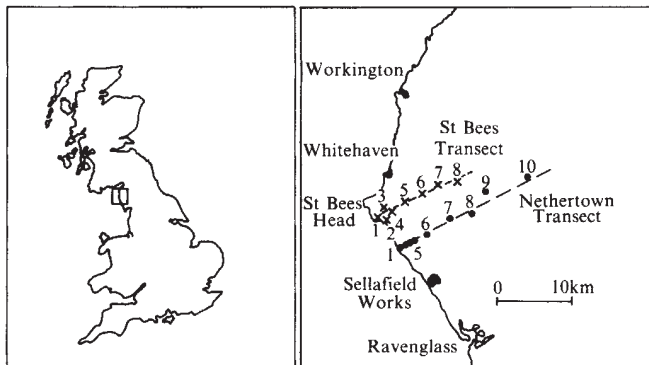


Fig. 1 Location of soil sampling sites.

Table 1 Deposits of ^{137}Cs , ^{238}Pu , $^{239+240}\text{Pu}$ and ^{241}Am in soil in West Cumbria

St Bees to Ennerdale (1977)						Nethertown to Newlands (1979)					
Site no.	Distance from sea (km)	Estimated rainfall (mm yr ⁻¹)	^{137}Cs	^{238}Pu	$^{239+240}\text{Pu}$	Distance from sea (km)	Estimated rainfall (mm yr ⁻¹)	^{137}Cs	^{238}Pu	$^{239+240}\text{Pu}$	^{241}Am
1	0.02	975	9	0.19	1.38	0.05	1,000	11.0	0.22	1.56	1.30
2	0.25	990	11	0.09	0.72	0.20	1,000	10.2	0.12	1.03	0.80
3	2.0	1,100	15	0.03	0.48	0.50	1,000	8.9	0.10	0.96	0.56
4	2.5	1,140	12	0.04	0.68	0.90	1,000	7.4	0.05	0.81	0.40
5	7.0	1,430	16	0.02	0.41	1.60	1,050	11	0.04	0.59	0.21
6	10.0	1,630	13	0.02	0.31	5.1	1,450	12	0.04	0.47	0.14
7	12.5	1,800	16	<0.01	0.18	8.0	1,800	15	0.02	0.61	0.11
8	13.75	1,800	15	0.02	0.29	10.9	2,000	9.6	0.03	0.51	0.10
9	—	—	—	—	—	14.3	2,350	25	0.02	0.51	0.13
10	—	—	—	—	—	20.5	2,130	19	0.02	0.41	0.10

Values are pCi cm⁻² to a depth of 15 cm.

The uncertainties, 2σ , on the above results associated with counting statistics are ~30%, 25%, 10% and 15% for ^{137}Cs , ^{238}Pu , $^{239+240}\text{Pu}$ and ^{241}Am respectively. The variability of sampling, 2σ , is generally <25%.

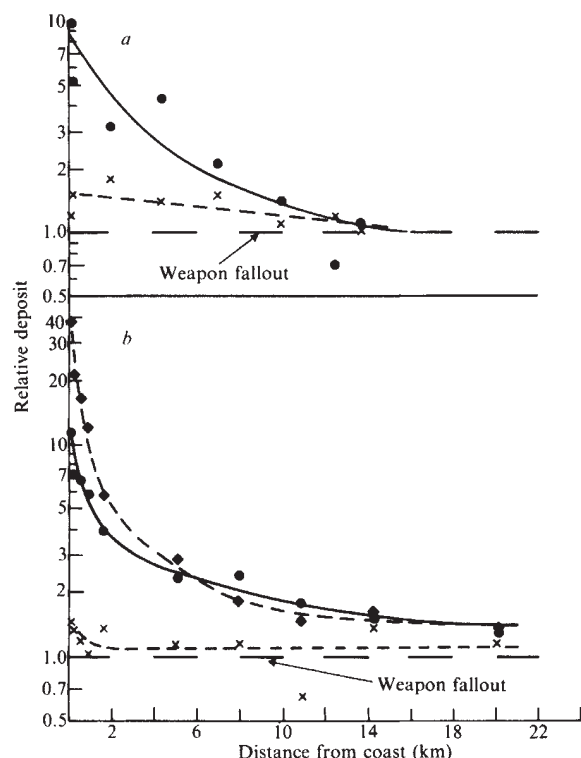


Fig. 2 Deposits of ^{137}Cs (x), $^{239+240}\text{Pu}$ (●) and ^{241}Am (◆) in soil, relative to fallout from nuclear weapons tests. *a*, St Bees Transect (1977); *b*, Nethertown Transect (1979).

using data published by the Meteorological Office¹⁷. In Fig. 2a the deposits of ^{137}Cs and $^{239+240}\text{Pu}$ at each site on the St Bees transect have been plotted, as a ratio to the estimated deposit due to fallout, against distance inland. Figure 2b shows similar results for the Nethertown transect, including data for ^{241}Am .

The $^{239+240}\text{Pu}$ deposit at the coast on both transects is about a factor of 10 greater than expected from nuclear weapons fallout. As may be seen from Table 1, the ratio $^{238}\text{Pu}/^{239+240}\text{Pu}$ falls with increasing distance from the coast. At the coast, the value of this ratio is characteristic of the highly irradiated material discharged from Sellafield^{5,6}. The relative deposit of ^{241}Am near the coast on the Nethertown transect is greater than that of $^{239+240}\text{Pu}$ but it decreases in a similar way with distance inland. In contrast, the deposit of ^{137}Cs in both transects is only slightly enhanced at the coast and rapidly becomes indistinguishable from nuclear weapons fallout.

Figure 3 shows the excess deposits of ^{238}Pu and $^{239+240}\text{Pu}$ on the St Bees transect plotted against distance from the coast.

Included in Fig. 3 are values for the annual sodium deposit derived from measurements at several sites in the United Kingdom^{18,19} and in Holland¹⁹. The latter agree with data reported elsewhere²⁰. It is apparent that the slopes of the three lines are broadly similar, demonstrating a correlation between Na and Pu deposition. This, together with the general shape of the deposition curves, is taken as good evidence that the excess Pu is of maritime origin and associated with seaspray. A similar maritime effect has been reported²¹ in the coastal region of Normandy, France, near the fuel reprocessing works at La Hague.

The excess deposit of $^{239+240}\text{Pu}$ in soil at St Bees is 1.2 pCi cm⁻² and the annual Na deposit at the coast (10⁴ μg cm⁻² from Fig. 3) is equivalent to ~10⁻³ l cm⁻² of seawater. Approximately 70% of the Pu discharged to sea between 1957 and 1977 was discharged after 1970, when the concentration of $^{239+240}\text{Pu}$ in filtered inshore seawater was reported⁷ to be ~0.5 pCi l⁻¹. The expected annual deposit of $^{239+240}\text{Pu}$ from seaspray at the coast would therefore be ~5 × 10⁻⁴ pCi cm⁻², or 3 × 10⁻³ pCi cm⁻² over a 6 yr period. An enrichment factor of 2 or 3 orders of magnitude in seaspray is therefore required to account for the excess $^{239+240}\text{Pu}$ in soil along the St Bees transect. An analysis of ^{241}Am deposits in the Nethertown transect indicates that a similar enrichment factor is required to account for the deposition of this radionuclide. However, the deposition of ^{137}Cs in both transects can be accounted for by levels in bulk seawater with no evidence of enrichment.

Possible mechanisms which may be involved in the transfer of radioactivity from sea to land are: (1) direct suspension of the sea surface by the wind in the form of spray; (2) conversion of the sea surface material into aerosol by bubble bursting^{22,23},

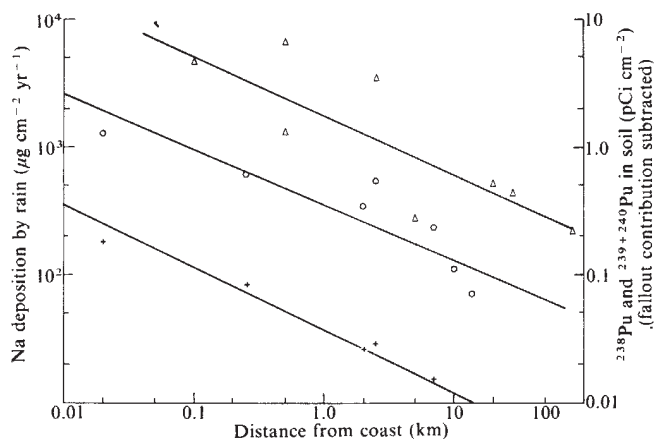


Fig. 3 Deposition of Na (Δ), ^{238}Pu (+) and $^{239+240}\text{Pu}$ (○) versus distance from the sea (1977).

(3) injection of seawater and suspended sediment into the air by waves breaking in the surf zone; and (4) movement of sediment to intertidal regions with subsequent resuspension by wind. Of themselves, the results do not favour any one of the above mechanisms but the apparent existence of an enrichment effect for Pu and Am in seaspray, and its absence for ^{137}Cs , suggests that the mechanism may be related to the sediment in seawater rather than to material in solution. Perhaps the most likely mechanism is the production of high concentrations of suspended particulates in the surf zone produced by the action of breaking waves and the consequent injection of particulates into the atmosphere with spray.

To put the observed values in perspective in relation to hazards they can be compared with some proposed limiting values. The highest observed deposit of Pu was $\sim 2 \text{ pCi cm}^{-2}$ in a 15 cm depth of soil. This is more than a factor of 1,000 below the Generalized Derived Limit for Pu in well-mixed soil as specified by the National Radiological Protection Board²⁴.

This work was supported in part by British Nuclear Fuels Ltd and the Department of the Environment.

Received 21 June; accepted 1 September 1982.

1. Howells, H. *Proc. Symp. on Disposal of Radioactive Wastes into Seas, Oceans and Surface Waters* (IAEA, Vienna 1966).
2. Hansard (7 April 1977).
3. Windscale Inquiry, BNFL document no. 208 (1977).
4. A. Rep. on Radioactive Discharges and Monitoring of the Environment, 1977 (BNFL, Risley, 1978).
5. A. Rep. on Radioactive Discharges and Monitoring of the Environment, 1978 (BNFL, Risley, 1979).
6. A. Rep. on Radioactive Discharges and Monitoring of the Environment, 1979 (BNFL, Risley, 1980).
7. Hetherington, J. A., Jefferies, D. F. & Lovett, M. B. *Proc. Conf. Impacts of Nuclear Releases into the Aquatic Environment* (IAEA, Vienna, 1975).
8. Hetherington, J. A., Jefferies, D. F., Mitchell, N. T., Pentreath, R. J. & Woodhead, D. S. *Proc. IAEA Symp. Transuranium Nuclides in the Environment*, San Francisco (1976).
9. Salmon, L. *Proc. Radiochemical Methods of Analysis*, 125 (IAEA Vienna, 1965).
10. Salmon, L. & Booker, D. V. *AERE Rep. R8870* (HMSO, London, 1978).
11. Lally, A. E. & Eakins, J. D. *Symp. on the determination of Radionuclides in Environmental and Biological Materials* (CEGB, London, 1978).
12. Peirson, D. H. & Salmon, L. *Nature* **184**, 1678–1679 (1959).
13. Peirson, D. H. & Cambray, R. S. *Nature* **205**, 433–440 (1965).
14. Cawse, P. A. *AERE Rep. AERE-PR-EMS 5* (1978); *AERE-PR-EMS 6* (1979).
15. Hardy, E. P., Krey, P. W. & Volchok, H. L. *Nature* **241**, 444–445 (1973).
16. Krey, P. W. *et al.*, *Proc. Conf. Transuranium Nuclides in the Environment*, San Francisco, 671–677 (1976).
17. *Rainfall Annual Average 1916–1950* (Ordnance Survey, London, 1967).
18. Cawse, P. A. *AERE Rep. AERE R7669* (1974).
19. Cambray, R. S., Jefferies, D. F. & Topping, G. *AERE Rep. AERE-R7733* (HMSO, London, 1975).
20. Malloch, A. J. C., *J. Ecol.* **60**, 103–112 (1972).
21. Frazier, A., Masson, M. & Guary, J. C. *J. Rech. Atmos.* **11**, 1 (1977).
22. Piotrowicz, S. R., Ray, B. J., Hoffman, G. L. & Duce, R. A. *J. geophys. Res.* **77**, 5243–5254 (1972).
23. Pattenden, N. J., Cambray, R. S. & Playford, K. *Geochim. cosmochim. Acta* **45**, 93–100 (1981).
24. Simmonds, J. R., Harrison N. T. & Linsley G. S. *Generalized Derived Limits for Radioisotope of Plutonium* (NRPB-DL5, 1982).

CH₄, H₂, CO and N₂O in submarine hydrothermal vent waters

Marvin D. Lilley, Marie A. de Angelis & Louis I. Gordon

School of Oceanography, Oregon State University, Corvallis, Oregon 97331, USA

Hydrothermal circulation systems of mid-ocean ridges profoundly influence the chemistry of the oceans and the oceanic crust^{1–3}. This has been demonstrated for several major and minor constituents of seawater⁴ and trace metals⁵. In addition, several volatile compounds including helium^{6,7} as well as methane and hydrogen^{8–11} are introduced to the sea floor in concentrations greatly exceeding that of ambient bottom water. We present here our measurements of concentrations of

methane, hydrogen, carbon monoxide and nitrous oxide in the hydrothermal vent waters from the Galapagos Spreading Centre (GSC). The relationships of these constituents to silicon were unique for each vent field, with nitrous oxide at East of Eden being the only instance of negative correlation. These gases could serve as important energy sources, in addition to hydrogen sulphide, for the chemosynthetic bacteria which support the extensive and diverse animal population living in these environments^{12,13}.

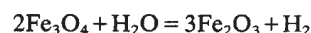
Two principal types of hydrothermal vents have been discovered and studied to date. At the GSC (0°48' N, 86°0.8' W) warm (7–23 °C) water flowed from gaps, or 'vents', between basaltic pillows at linear flow rates of the order of cm s^{-1} ; similar vents have been found at 21° N on the East Pacific Rise^{14,15}. The second type, found at 21° N but not at the GSC, had hot (~350 °C) water flowing from sulphide 'chimneys' at rates of m s^{-1} . The data presented here represent hydrothermal water samples collected using the research submersible, *Alvin*, from three separate vent fields at the GSC in March 1979.

Corliss *et al.*¹ showed that the silica concentration in the GSC vent samples defined a single linear relationship with temperature and, owing to uncertainties in the temperature measurements, provided a better measure of the degree of mixing between the hydrothermal end-member and ambient seawater than did temperature. We therefore present the GSC gas data relative to dissolved silica in Fig. 1.

CH₄/³He and H₂/³He ratios for these vents are compared with those from sites along the East Pacific Rise in Table 1. The 21° N hydrothermal end-member is chemically very similar to the Galapagos end-member estimated by magnesium and silica geothermometry³.

If the hydrothermal end-member solutions were in equilibrium with the quartz-fayalite-magnetite (QFM) buffer then they should contain approximately 400 mM hydrogen at 350 °C and 500 bar (ref. 18). This would imply H₂/³He ratios of the order of 10¹⁰ which are orders of magnitude higher than those observed (Table 1). Either the QFM buffer system does not control the H₂ concentration in these solutions or significant H₂ sinks, such as reduction of CO₂ and sulphate, occur in the ascending fluids. Hydrogen is an excellent energy source for several species of chemosynthetic bacteria¹⁹, hence these reductions could take place abiotically or microbially in the warm water vents.

All of the reduced gases discussed here are produced both abiotically and microbially. Possible abiotic sources of methane, carbon monoxide and hydrogen include injection from the mantle and extraction from basalt by hot fluids. Additional abiotic sources include the reduction of carbon dioxide to form methane and the oxidation of magnetite to haematite,



which has been postulated as the major source of volcanic hydrogen²⁰. Nitrous oxide could be produced abiotically by the reduction of nitrate in the descending seawater or by the oxidation of ammonium ascending with the hydrothermal fluids. Ammonium is known to substitute for alkali metal ions in silicate minerals and to be stable at high temperatures and pressures²¹. Nitrous oxide has also been detected in a few volcanic gas samples²². Carbon monoxide is a common constituent of volcanic gases²³ and could originate from C–O–H–S equilibria within the magma²⁴.

An alternative source of these gases in the hydrothermal fluids could be biological production. It is well established that methane, carbon monoxide, hydrogen and nitrous oxide are produced and consumed by microorganisms²⁵. Hydrogen can be produced by many microbial species, methane by only one group, the methanogens. Little is known about the production of carbon monoxide by microorganisms but there is evidence that carbon monoxide may be an intermediate in methane oxidation²⁶.

Nitrous oxide is produced microbially through nitrification and denitrification. Both nitrifying and denitrifying bacteria

have been isolated from water samples and animal guts taken from the GSC and 21° N vents (J. Baross, personal communication). Although nitrification and denitrification usually do not take place in sulphide bearing waters such as the vent waters, some bacteria, for example, *Thiobacillus denitrificans*²⁷, can reduce nitrate to nitrous oxide using sulphide as the electron donor. The nitrogen isotope data²⁸ indicate that the nitrogen found in vent animals is depleted in ¹⁵N and its assimilation into animal tissue must have been preceded by bacterial nitrogen fixation and/or the bacterial assimilation of nitrate or ammonium.

We now consider the observation that nitrous oxide concentrations were found to be enhanced in some and depleted in other GSC vent waters compared with the ambient waters. In the ambient seawater, oxygen and nitrate are the principal electron acceptors involved in biological oxidation. Convective circulation within the warm water vent systems involves penetration of ambient water, thereby supplying these electron acceptors to the resident chemolithotrophic population^{1,12,13}. In the process of nitrate reduction, nitrite and nitrous oxide can accumulate and/or serve as intermediate electron acceptors. East of Eden which had the highest observed sulphide to silica ratio (0.853) had the greatest residual potential for reducing electron acceptors. This is consistent with East of Eden nitrous oxide concentrations being lower than those of ambient seawater. Mussel Bed and Rose Garden had lower sulphide to silica ratios (0.072 and 0.232, respectively) and showed net

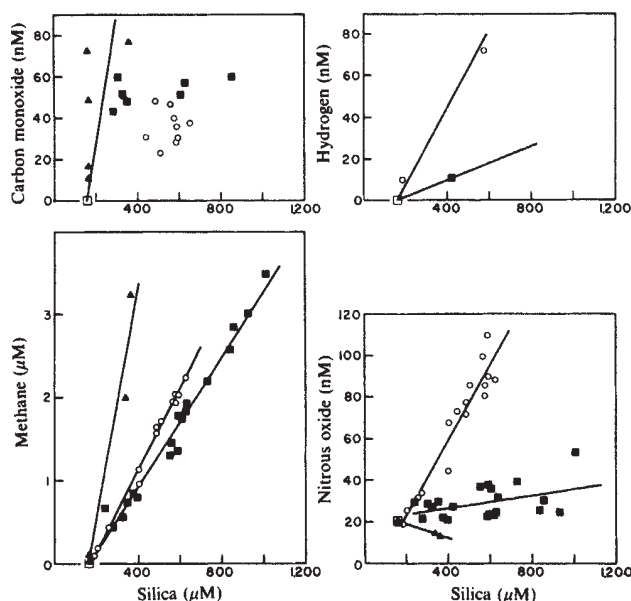


Fig. 1 Reduced gas correlations with silica for the GSC data. The locations and equations for the regression lines shown (not forced through the ambient seawater conditions) are:

△ East of Eden

$$\text{CH}_4 = -2.23 + 0.0140 \text{ Si} \quad \text{N}_2\text{O} = 25.6 - 0.034 \text{ Si}$$

○ Mussel Bed

$$\text{CH}_4 = -0.82 + 0.0049 \text{ Si} \quad \text{N}_2\text{O} = -12.0 + 0.178 \text{ Si}$$

■ Rose Garden

$$\text{CH}_4 = -0.63 + 0.0039 \text{ Si} \quad \text{N}_2\text{O} = 19.8 + 0.016 \text{ Si}$$

The regression line for CO is drawn for the East of Eden data only. Ambient bottom water concentrations were: CH₄, 0.3 nM; N₂O, 20.8 nM; CO, 0.3 nM; H₂, 0.4 nM. Each vent field was characterized by a different range of water temperatures and unique H₂S/Si mole ratios, namely △, 7–8 °C, 0.853; ○, 8–13 °C, 0.072; ■, 8–23 °C, 0.232 (J. Edmond, personal communication). All water samples, except those for H₂ which were taken in PVC samplers, were collected with the pumping system described by Corliss *et al.*¹. These were subsampled into 125 ml tubular Pyrex flasks with Teflon stopcocks at either end and poisoned to 0.4 mM with HgCl₂. A modified version of the gas chromatographic procedure of Swinnerton and Linnenbom¹⁶ was used. The gas chromatograph was equipped with helium ionization and flame ionization detectors.

Table 1 Ratios of hydrogen and methane to ³He

Vent field	CH ₄ / ³ He (mole ratio)	H ₂ / ³ He (mole ratio)
Galapagos*		
East of Eden	42.0 ± 10.9 × 10 ⁶	—
Mussel Bed	14.8 ± 1.8 × 10 ⁶	0.7 ± 0.2 × 10 ⁶
Rose Garden	12.4 ± 1.1 × 10 ⁶	0.1 × 10 ⁶
East Pacific Rise		
21° N, 109° W†	5.0 × 10 ⁶	22–43 × 10 ⁶
20° S, 114° W‡	5.8–17.4 × 10 ⁶	—

* ³He data for these GSC vents are not available. We assumed that ³He in these vents does not differ appreciably from the values obtained by Jenkins *et al.*⁷ for the GSC vents sampled during the 1977 expedition. ³He values for our samples were derived by combining the ³He-temperature relationship from their Fig. 2 (using the slope which included Garden of Eden) and the temperature-Si relationship for our samples: $T (^{\circ}\text{C}) = \frac{\text{Si}(\mu\text{M}) - 28}{59.77}$ (J. Edmond, personal communication).

† Data from ref. 9.

‡ Data from ref. 17.

production of nitrous oxide. The determining factor for net production or consumption of nitrous oxide is probably the local redox potential history of a water parcel within the more shallow convection cells of a given vent system.

In addition to the variations seen in reduced gas concentrations among vent fields, systematic variations in several other chemical species have been reported in the GSC vent fields^{1,3,5}. These may result from several causes, such as vent field age, differing substrate compositions and differences in water/rock ratios⁴. We hypothesize that the composition of the microbial assemblage in each vent field is characteristic of that field and this is reflected in characteristic production and consumption rates of the gases and other biogenous constituents in the vent water. This hypothesis is supported by the recent discovery that mixed-culture microbial populations, isolated from hot water and chimney rock samples taken at 21° N, can produce hydrogen, methane, carbon monoxide and nitrous oxide and consume methane at 100 °C (ref. 29).

Methane ¹³C data indicate that the 21° N methane was abiogenic in origin³⁰ but ¹³C measurements have not been done on GSC methane. However, because the CH₄/³He ratio at 21° N is considerably less than that in the cooler GSC vents, and as methanogens are present in vent samples at 21° N (ref. 29), we conclude that methanogens are likely present in the warm Galapagos vents and that part of the methane found in these vents is microbially produced. This is most clearly indicated at East of Eden which had the highest CH₄/³He ratio (Table 1) yet measured in these vent systems¹⁷. This conclusion is consistent with the reduced hydrogen levels in the GSC vents; hydrogen would be consumed in the microbial production of methane. Indeed, the question of possible microbial effects on the chemical constituents of submarine hydrothermal systems needs closer scrutiny.

Support for this research was provided by the Office of Naval Research, Marine Chemistry Program, under contract N00014-79-C-0004. We thank the officers and crews of *Alvin*, *Lulu*, and *Gilliss* for assistance, J. Edmond for use of unpublished data, and J. Baross for stimulating discussion throughout, also Erwin Suess and Harmon Craig for helpful reviews.

Received 18 June; accepted 6 September 1982.

- Corliss, J. B. *et al. Science* **203**, 1073–1083 (1979).
- East Pacific Rise Study Group *Science* **213**, 31–40 (1981).
- Edmond, J. M., Von Damm, K. L., McDuff, R. E. and Measures, C. F. *Nature* **297**, 187–191 (1982).
- Edmond, J. M. *et al. Earth planet. Sci. Lett.* **46**, 1–18 (1979).
- Edmond, J. M. *et al. Earth planet. Sci. Lett.* **46**, 19–30 (1979).
- Lupton, J. E., Weiss, R. F. & Craig, H. *Nature* **267**, 603–604 (1977).
- Jenkins, W. J., Edmond, J. M. & Corliss, J. B. *Nature* **272**, 156–158 (1978).
- Welhan, J. A. & Craig, H. *Geophys. Res. Lett.* **6**, 829–831 (1979).
- Lilley, M. D. & Gordon, L. I. *EOS* **46**, 863 (1979).
- Welhan, J. A. thesis, Univ. California, San Diego (1981).
- Welhan, J. *EOS* **61**, 966 (1980).
- Karl, D. M., Wirsén, C. O. & Jannasch, H. W. *Science* **207**, 1345–1347 (1980).
- Jannasch, H. W. & Wirsén, C. O. *Appl. envir. Microbiol.* **41**, 528–538 (1981).

14. Cyamex Scientific Team *Nature* **277**, 523–528 (1979).
15. Rise Project Group *Science* **207**, 1421–1432 (1980).
16. Swinnerton, J. W. & Linnenbom, V. J. *J. chromat. Sci.* **5**, 570–573 (1967).
17. Craig, H. *Eos* **62**, 893 (1981).
18. Wolery, T. J. & Sleep, N. R. *J. Geol.* **84**, 249–275 (1976).
19. Zajic, J. E., Kosaric, N. & Brosseau, J. D. *Adv. biochem. Engng* **9**, 57–109 (1978).
20. Arrhenius, G. *Adv. Space Res.* **1**, 37–48 (1981).
21. Hallam, M. & Eugster, H. P. *Contrib. miner. Petrol.* **57**, 227–244 (1976).
22. Cicerone, R. J., Shetter, J. D., Stedman, D. H., Kelly, T. J. & Liu, S. C. *J. geophys. Res.* **83**, 3042–3050 (1978).
23. Gerlach, T. M. *J. volcanol. Geotherm. Res.* **8**, 177–189 (1980).
24. Gerlach, T. M. & Nordlie, B. E. *Am. J. Sci.* **275**, 353–376 (1975).
25. Cole, J. A. *Adv. microbiol. Phys.* **14**, 1–92 (1976).
26. Baross, J. A., Lilley, M. D., Dahm, C. N. & Gordon, L. I. *EOS* **63**, 155 (1982).
27. Aminuddin, M. & Nicholas, D. J. D. *Biochim. biophys. Acta* **325**, 81–93 (1973).
28. Rau, G. *Nature* **289**, 484–485 (1981).
29. Baross, J. A., Lilley, M. D. & Gordon, L. I. *Nature* **298**, 366–368 (1982).
30. Welhan, J., Kim, K. & Craig, H. *EOS* **62**, 913 (1981).

Palaeomagnetism of volcanic rocks of the Kodiak Islands indicates northward latitudinal displacement

Peter W. Plumley*, Robert S. Coe*, Tim Byrne†, Mary R. Reid‡ & J. Casey Moore*

* Earth Sciences Board, University of California, Santa Cruz, California 95064, USA

† Department of Geology, Cornell University, Ithaca, New York 14853, USA

‡ Department of Earth and Planetary Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

In the past decade it has become widely accepted that southern Alaska is composed of allochthonous terranes^{1–4}, but the times of amalgamation and their accretion to North America remain controversial. Palaeomagnetists and geologists generally agree that the Talkeetna superterrane, consisting of the Wrangellia and Peninsular terranes (Fig. 1), was far to the south of its present position with respect to North America in Triassic and Jurassic time^{1–4}. This consensus is lost when it comes to the question of the relative position of these terranes in the Tertiary. Previously, limited palaeomagnetic evidence suggested that the Peninsular terrane may have lain substantially south of its present position⁴, but the timing of deformational and thermal events and the apparent absence of a Tertiary suture zone argue for a Cretaceous docking of Wrangellia and the Peninsular terrane^{5,6}. Here we present new palaeomagnetic results demonstrating that the Kodiak Islands were indeed at a more southerly latitude 62 Myr ago, around 43° N, and discuss the implications of this conclusion.

We sampled the volcanic rocks of the Palaeocene Ghost Rocks Formation exposed along the southeastern margin of the Kodiak Islands (Fig. 1). This turbidite sequence consists of a landward belt with intercalated flows of andesite pillows and a seaward belt including basalt flows. The sedimentary rocks are commonly isoclinally folded and locally grade into zones of tectonic disruption. The volcanic rocks are generally much less deformed and typically form coherent folds. The Ghost Rocks Formation is part of a late Cretaceous to Palaeogene accretionary sequence. The formation is apparently thrust to the north-west against a late Cretaceous trench turbidite sequence⁷ and underthrust from the south-east by an Eocene to Oligocene submarine fan complex⁸. Metamorphic prehnite, pumpellyite and laumontite are locally developed⁹. The youngest foraminifera (Palaeocene) and 62 Myr radiometric ages of cross-cutting plutons suggest an early Palaeocene depositional age for the Ghost Rocks Formation.

We drilled and oriented cores of Ghost Rocks volcanics from two regions on Kodiak Island—Kiliuda Bay and Alitak Bay—which are separated by ~80 km along strike. In all, an average of seven cores were collected from each of 29 flows in the two regions. Flow attitudes were almost always obtained from interbedded sediments, and only occasionally from pillow mor-

phology. The Kiliuda Bay sites included locations in both the andesite belt and the basalt belt, whereas the Alitak Bay sites included flows from the andesite belt only.

Stepwise thermal demagnetization proved effective in cleaning the samples from almost all of the flows. Characteristic components of natural remanent magnetization were isolated at temperatures of 300 °C and higher that trended towards the origin on vector diagrams. Considerable improvement in clustering of directions resulted for most flows: the average *k* increased from 15 to 40 during thermal cleaning. Only 2 of the 29 sites failed to clean up, and one of these was a highly deformed, badly fractured tuff.

Both polarities were found in the flows collected at Kiliuda Bay. Application of the *F*-test shows that normal and reverse mean directions are not significantly different from antiparallel. These directions also pass the fold test: when correction is made for the tectonic dip of the flows, *k* increases from 4 in geographical coordinates to 29 in stratigraphical coordinates (Table 1). At Alitak Bay only reversely magnetized flows are present, but these also pass the fold test (Table 1). Thus there is strong evidence that the characteristic magnetization of the rocks pre-dates folding in both areas and probably preserves the original field direction. Even after tectonic correction, however, the mean directions for the two regions are very different (Table 1). The mean declinations differ by 97°, suggesting the regions may have been rotated with respect to each other about a vertical axis. The inclinations also differ from each other by a much smaller, but still statistically significant amount. A likely explanation for this is that the Alitak Bay group of flows spans insufficient time to average out secular variation, although other explanations involving systematic differences in initial dips or in ages of flows between the two regions cannot be categorically ruled out.

The most important palaeomagnetic parameter for regional-scale tectonic interpretation is the mean inclination of the Palaeocene geomagnetic field for the Kodiak Islands. It is reasonable to assume that some sort of average of the data sets for the two regions would give a better estimate of the true inclination than either one of them alone. There are several techniques that have been developed for averaging inclinations only, when declination data are unavailable or should be excluded because of scatter (refs 10, 11, and A. Cox and R. Gordon, in preparation). A straightforward and simple way to do it for this case, however, is to rotate the sets of directions for both regions so that their mean declinations coincide (Fig. 2). Note that the resulting distribution of directions shown in Fig. 2 does not appear particularly non-fisherian (certainly it is not bimodal), which supports the idea that the discrepancy in inclinations between the two regions may be due to incomplete averaging of secular variation. Analysis of the combined

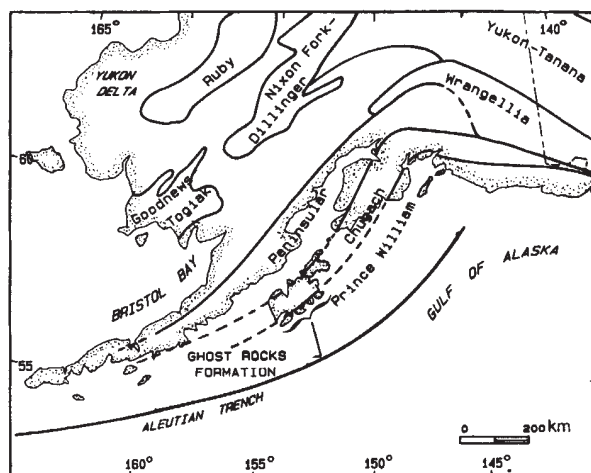


Fig. 1 Generalized terrane map of southern Alaska¹⁴ showing location of the Ghost Rocks Formation.

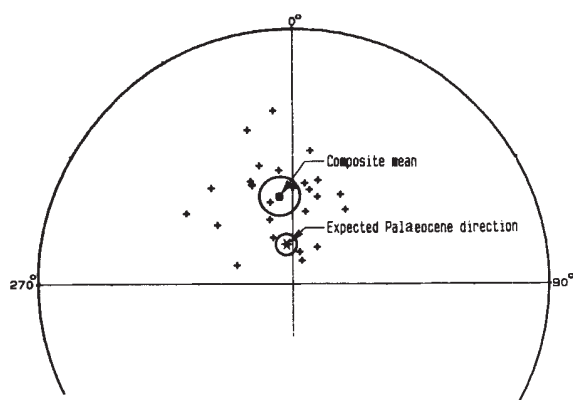


Fig. 2 Site mean directions (reversed inverted to normal): the mean declination for each region (Kiliuda Bay, Alitak Bay) has been rotated (31° clockwise, and 65° anticlockwise respectively) to coincide with the expected Palaeocene declination. Mean of composite data set (■) and expected Palaeocene direction (★) are shown with associated 95% confidence limits.

data set by Fisher statistics¹² gives a mean inclination of 61.4° and 95% confidence limits of $\pm 6.5^\circ$. The same mean inclination, within a few tenths of a degree, is obtained by any of the more sophisticated inclination or colatitude statistics referred to above.

As shown in Fig. 2, the mean inclination of the Ghost Rocks volcanics is significantly shallower than that expected for Kodiak if it had been in its present position with respect to North America in Palaeocene time. The corresponding palaeolatitude is $43 \pm 9^\circ$ N, which is significantly less than the expected value of $66 \pm 6^\circ$ N derived from Palaeocene palaeomagnetic data for cratonic North America¹³. Thus the latitudinal displacement of Kodiak with respect to North America that is indicated is $23 \pm 11^\circ$ northward (Fig. 3); any longitudinal displacement is, of course, indeterminate.

The lithology, structural style and geological setting of the Ghost Rocks Formation suggest that it is a Palaeocene subduction complex. On the tectonostratigraphic map of Jones *et al.*¹⁴ (Fig. 1) it is classified as part of the Prince William terrane. However, it is tied to the adjacent Chugach terrane (Fig. 1) by plutons of identical age (60–62 Myr) and composition. The only published palaeomagnetic data from the Chugach terrane are from two Eocene layered-gabbro plutons and a Cretaceous sheeted dyke and pillow basalt complex studied by Grommé and Hillhouse¹⁵. Although no fold test was described, their results from both sites suggest the Chugach terrane was about 25° south of its present position with respect to North America at these times, in excellent agreement with our results from the Prince William terrane.

The next terrane inboard is the Peninsular terrane (Fig. 1). It is simplest to suppose that the Peninsular terrane formed the backstop against which the late Cretaceous–early Tertiary accretionary complex of the Chugach–Prince William terranes formed, although the geological evidence for this is equivocal.

Table 1 Palaeomagnetic results for Ghost Rocks volcanics

Kiliuda Bay					
	Dec	Inc	A95	k	N
Geographical	345.5	61.9	25.9	4.1	11*
Stratigraphical	320.1	52.7	8.5	29.5	11*
Alitak Bay					
Geographical	168.2	36.0	12.6	10.9	14†
Stratigraphical	56.7	68.3	8.6	22.6	14†
Ghost Rocks					
Mean	351.4	61.4	6.5	21.3	25

* Two sites eliminated because of unstable magnetization.

† Two superposed flows with identical NMR eliminated because directions were grossly discordant (>2 s.d. from mean).

Certainly very similar accretionary processes are occurring there today. The relatively few Tertiary palaeomagnetic data that have been published for the Peninsular terrane are consistent with this idea. In a study of 74 cores of lower Tertiary sediments from four closely spaced localities near Pavlov Bay, Stone and Packer⁴ found shallow inclinations that imply low palaeolatitudes comparable to those we have found for the Ghost Rocks Formation.

Much further inboard, just north of the Denali fault, one finally encounters concordant palaeomagnetic directions. In a study of the Teklanika volcanics, Hillhouse and Grommé¹⁶ have found steep inclinations consistent with the expected palaeocene field. Our own work on Ordovician limestones and a few early Tertiary lava flows in the Nixon Fork terrane 80 km to the west is consistent with their finding¹⁷. Thus, according to the palaeomagnetic evidence, considerable Tertiary convergence must have been taken up somewhere south of the Denali fault.

Figure 3 shows the band of possible Palaeocene positions for the southern terranes compatible with the palaeomagnetic results for the Ghost Rocks Formation. We have chosen our study to position the southern terranes in the early Tertiary because it is the only one on igneous rocks of well determined age and palaeohorizontal control that passes fold and reversal tests. The locations of the Pacific, Farallon and North American plate boundaries are essentially those of Engebretson (in preparation), who assumes fixed hotspots. The position of the Kula–Farallon ridge is less well constrained because the relevant magnetic anomalies have been subducted.

We favour a Palaeocene position of the southern terranes near to the Kula–Farallon ridge as drawn in Fig. 3 because other evidence suggests that the Ghost Rocks volcanics were the product of a ridge–trench encounter. These lavas are much too close to the subduction zone to be normal arc volcanism^{18,19}, and their composition, as well as that of the correlative pluton, suggests that they all have a mid-ocean ridge basalt parent^{20,21}. Positioning the southern terranes north of the Kula–Farallon ridge allows them to be pushed northward by the movement of the Kula and Pacific plates, rather than being diverted to

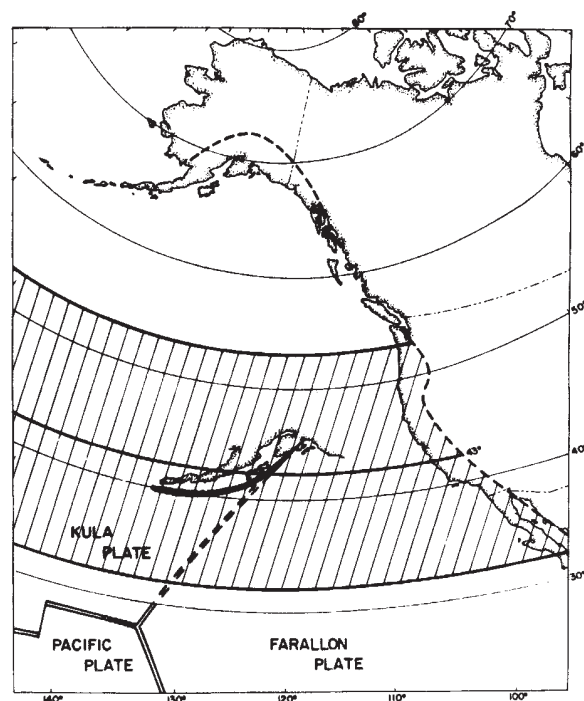


Fig. 3 Palaeocene reconstruction showing palaeomagnetically inferred latitude for Ghost Rocks Formation, Kodiak, Alaska. Stippled palaeolatitudinal band is the uncertainty with respect to North America at the 95% confidence level. Heavy dashed lines suggest the Palaeocene North American plate margin.

the east by the motion of the Farallon plate. The mystery of the missing Tertiary suture zone in central Alaska required by this reconstruction, however, remains an enigma. These ideas and the data on which they are based will be presented in full detail elsewhere.

This investigation was supported by NSF grants EAR77-07836, EAR80-08213 and EAR80-07464. Additional support from the following companies has been greatly appreciated: Mobil Oil Corporation, Amoco Production Co., Union Oil, Exxon USA, and the US Geological Survey.

Received 20 April; accepted 16 June 1982.

1. Jones, D. L., Silberling, N. J. & Hillhouse, J. W. *Can. J. Earth Sci.* **14**, 2565–2577 (1977).
2. Hillhouse, J. W. *Can. J. Earth Sci.* **14**, 2578–2592 (1977).
3. Coney, P. J., Jones, D. L. & Monger, J. W. H. *Nature* **288**, 329–333 (1980).
4. Stone, D. B. & Packer, D. R. *Tectonophysics* **37**, 183–201 (1977).
5. Csejtei, B. Jr & St Aubin, D. R. *U.S. geol. Surv. Circ.* **823-B** B49–B51 (1981).
6. Silberman, M. L., MacKevett, E. M. Jr, Connor, C. L., Klock, P. R. & Kalechitz, G. *U.S. geol. Surv. Circ.* **823-B** B61–B63 (1981).
7. Moore, G. W. *U.S. geol. Surv. Bull.* **1274-A**, A27–A35 (1969).
8. Nilsen, T. H. & Moore, G. W. *U.S. geol. Surv. Prof. Pap.* **1093** (1979).
9. Byrne, T. in *Trench–Forearc Geology: Sedimentation and Tectonics on Modern and Ancient Active Plate Margins* (ed. Leggett, J. K.) 229–242 (Blackwell, Oxford, 1982).
10. Briden, J. C. & Ward, M. A. *Pure appl. Geophys.* **63**, 133–152 (1966).
11. Kono, M. *J. geophys. Res.* **85**, 3878–3882 (1980).
12. Fisher, R. A. *Proc. R. Soc. A27*, 295–305 (1953).
13. Jacobson, D., Beck, M. E. Jr, Diehl, J. F. & Hearn, B. C. *Geophys. Res. Lett.* **7**, 549–552 (1980).
14. Jones, D. L., Silberling, N. J., Berg, A. C. & Plafker, G. *U.S. geol. Surv. Open-File Rep.* **81-792**, 1–20 (1981).
15. Grommé, C. S. & Hillhouse, J. W. *U.S. geol. Surv. Circ.* **823-B**, B70–B72 (1981).
16. Hillhouse, J. W. & Grommé, C. S. *EOS* **62**, 854 (1981).
17. Plumley, P. W. & Coe, R. S. *Geol. Soc. Am. Abstr. Prog.* **14**, 224 (1982).
18. Marshak, R. S. & Karig, D. E. *Geology* **5**, 233–236 (1977).
19. Byrne, T. *Geology* **7**, 341–344 (1979).
20. Reid, M. R. & Gill, J. B. *Geol. Soc. Am. Abstr. Prog.* **12**, 148 (1980).
21. Hill, M., Morris, J. & Whelan, J. J. *geophys. Res.* **86**, 10569–10590 (1981).

$^{40}\text{Ar}/^{39}\text{Ar}$ dating of pyrite

Derek York, A. Masliwec, P. Kuybida, J. A. Hanes*, C. M. Hall, W. John Kenyon, E. T. C. Spooner† & S. D. Scott†

Department of Physics, and †Department of Geology, University of Toronto, Toronto, Ontario, Canada M5S 1A7

The precise determination of the age of sulphide ore deposits is one of the most difficult problems in geochronology. This is largely because the very process responsible for concentrating certain metals to an economically significant level also tends to exclude radioactive isotopes from the deposits. It is customary to try to determine the age of mineralization by analysing silicate material presumed to be cogenetic¹. Because such materials are not always available, and because it is not always clear that the silicates and ore minerals are in fact contemporaneous, we examined the sulphide minerals themselves to see if they contained sufficient potassium and argon for $^{40}\text{Ar}/^{39}\text{Ar}$ age determination. Our initial results indicate that this is the case for pyrite from the Geco ore body in northwestern Ontario, Canada.

The direct dating of sulphides with the Rb–Sr technique was attempted by Reesman and Hurley². The results showed promise, but the approach was never pursued. More recently, Luck and Allègre³ have shown that some deposits may be datable with the Re–Os method. Most promising is the pioneering work on the Rb–Sr dating of fluid inclusions in quartz from a tungsten deposit by Shepherd and Darbyshire⁴.

Six pyrite samples have been analysed from the Geco mine, a stratabound and stratiform volcanogenic massive Cu–Zn sulphide deposit⁵. Geco has long been of interest to students of lead isotopes in connection with calculations of the age of the Earth and the chemical evolution of the mantle and crust⁶. The ore body has experienced the Kenoran orogeny⁵ and therefore

Table 1 Analytical data for Geco biotites (step-heating)

T (°C)	Atmospheric (%)	$^{40}\text{Ar}^*$ ($\times 10^{-5} \text{ cm}^3$ STP g ⁻¹)	f(39)	Age (Myr)
Sample GB250†				
810	1.2	6.21	3.7	2,530 ± 31
910	0.2	42.80	24.3	2,589 ± 3
1,100	0.2	55.51	30.8	2,620 ± 3
1,600	0.2	73.54	41.3	2,604 ± 2
Total	0.2	178.06	Integrated age	2,604 ± 5

Standard (40/39)_s = 43.57 ± 0.19

Mass = 0.0091g

Sample GB1050

550	52.8	0.08	0.2	1,137 ± 142
650	24.8	0.17	0.5	802 ± 104
700	5.9	0.88	0.5	2,426 ± 41
780	2.3	2.78	1.4	2,614 ± 8
820	0.4	21.94	11.5	2,600 ± 5
950	0.1	73.51	37.4	2,637 ± 3
1,170	0.1	89.78	45.8	2,633 ± 4
1,600	2.0	5.21	2.7	2,630 ± 11
Total	0.3	194.35	Integrated age	2,623 ± 6

Standard (40/39)_s = 45.22 ± 0.32

Mass = 0.0197g

Decay constants from ref. 11 were used. Errors are ±1σ.

† Only negligible volumes of $^{40}\text{Ar}^*$ were released from GB250 below 800 °C.

we had *a priori* information on its age. U–Pb and Rb–Sr dating in the area had shown⁷ that the last severe regional metamorphism occurred 2,600–2,700 Myr ago. Furthermore, we could add a much more specific age constraint to the ore body when, during mineral separation, we discovered minute amounts of the easily datable biotite in intimate association with sphalerite crystals. A few flecks of these micas were therefore hand-picked for age determination along with the six pyrite samples.

All samples were irradiated in the McMaster University reactor along with the Zartman hornblende standard⁸. Step-heating runs were performed on two biotite samples whilst the pyrites, because of their extremely low potassium concentrations (5–35 p.p.m.), were fused in single step analyses. The mass spectrometric isotope analysis of the evolved argon was carried out with an MS10 machine. The results are shown in Figs 1 and 2 and Tables 1 and 2. The biotite age spectra in Fig. 1 show excellent plateaus and there is evidence of only minute argon loss in the low temperature fractions. The plateaus are identical within experimental error and indicate that the cooling age of the ore body, after the Kenoran orogeny, is 2,610 ± 20 Myr. The age spectra indicate that the deposit has undergone negligible heating since then.

The pyrite data are shown in Fig. 2 where $^{40}\text{Ar}/^{36}\text{Ar}$ is plotted against $^{39}\text{Ar}/^{36}\text{Ar}$. Such a graphical analysis must, of course, be used as there is no knowing *a priori* what the $^{40}\text{Ar}/^{36}\text{Ar}$ composition was in the pyrites at the time they cooled below their argon retention temperatures. The data points in Fig. 2 are clearly strongly linearly correlated, but the scatter about a straight line exceeds that due solely to expected experimental error. The scatter cannot be accounted for by system blank

Table 2 Analytical data for Geco pyrites

Sample	Mass (g)	K (p.p.m.)	$^{40}\text{Ar}^*$ ($\times 10^{-7} \text{ cm}^3$ STP g ⁻¹)	$^{40}\text{Ar}/^{36}\text{Ar}$	$^{39}\text{Ar}/^{36}\text{Ar}$
OGS-10	0.2478	5.6	1.97	393.3 ± 4.5	0.34 ± 0.02
OGS-14	0.2613	8.8	2.56	444.4 ± 2.8	0.54 ± 0.01
OGS-131	0.8568	34.4	7.34	1,219.0 ± 9.0	5.13 ± 0.04
OGS-132	0.8484	16.8	3.72	840.9 ± 15.5	2.88 ± 0.02
OGS-133	0.8933	4.8	2.76	631.1 ± 7.6	0.68 ± 0.01
OGS-134	0.9348	11.9	3.06	521.4 ± 6.3	0.56 ± 0.01

Errors are ±1σ. $^{40}\text{Ar}/^{36}\text{Ar}$ and $^{39}\text{Ar}/^{36}\text{Ar}$ values have been corrected for a system blank of $1 \times 10^{-8} \text{ cm}^3$ STP. The system blank has atmospheric argon composition.

* Present address: Department of Geology, Queen's University, Kingston, Ontario, Canada K7L 3N6.

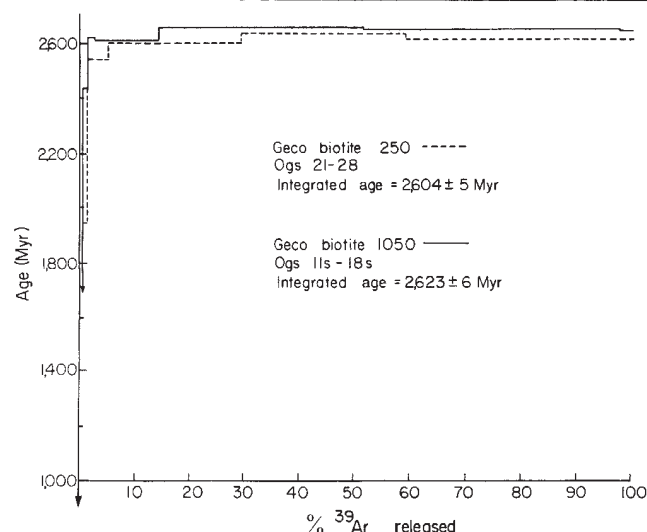


Fig. 1 Step heating data for Geco biotites. The age errors are listed in Table 1.

variation as the blank correction is no more than 5% of the plotted ratios. Thus enormous blank variations would have to be invoked for point OGS-134 (see Table 2).

When a least squares best line⁹ is fitted to all the data, the slope corresponds to an age of $2,490 \pm 200(1\sigma)$ Myr, with an initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of $369 \pm 28(1\sigma)$. When the most obviously discrepant point is omitted from the fit, the age and intercept estimates are scarcely changed, but the error estimates are significantly reduced. Thus the age becomes $2,500 \pm 120$ Myr, and the initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio is 359 ± 17 . The errors quoted in all cases correspond to the observed scatter of the points about the line.

The age indicated by the pyrites is thus indistinguishable, within errors, from that found for the micas, raising the distinct possibility of dating pyrite-bearing ore bodies directly with the $^{40}\text{Ar}/^{39}\text{Ar}$ technique despite the extremely low potassium

levels. The reason for the scatter of the data in Fig. 2 is not yet clear. However, it is probably a reflection of a lack of homogeneity in the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio among the pyrites at the onset of argon retention following the Kenoran orogeny.

At this stage, it is not possible to say where the potassium and argon are located in the samples. All the pyrites were carefully hand-picked and examined to exclude external silicate contamination and the extremely low potassium concentrations found (Table 2) are testimony to the efficiency of this. However, there may have been minute potassium-bearing silicate inclusions completely encased within the pyrite. Some potassium and argon could be present in fluid inclusions, although the chlorine/potassium ratios (0.5–1) estimated from the $^{38}\text{Ar}/^{39}\text{Ar}$ ratios were not those characteristic of fluid inclusions (≈ 45) (ref. 10).

This investigation was funded with grants from the Ontario Geological Survey and the National Science and Engineering Research Council of Canada.

Received 1 July; accepted 6 September 1982.

1. Kink, A. R. *Geochim. cosmochim. Acta* **29**, 717–724 (1965).
2. Reesman, R. H. & Hurley, P. M. *Earth planet. Sci. Lett.* **5**, 23–26 (1968).
3. Luck, J. M. & Allegre, C. J. *EOS* **62**, 430 (1981).
4. Shepherd, T. J. & Darbyshire, D. P. F. *Nature* **290**, 578–579 (1981).
5. Scott, S. D. & Sangster, D. F. in *The Handbook of Stratiform and Stratabound Ore-Deposits* Vol. 6, 159–164 (Elsevier, Amsterdam 1976).
6. York, D. & Farquhar, R. M. *The Earth's Age and Geochronology* (Pergamon, Oxford, 1972).
7. Tilton, G. R. & Steiger, R. H. *Science* **150**, 1805–1807 (1965).
8. Zartman, R. E. *J. Petrol.* **5**, 539–563 (1964).
9. York, D. *Earth planet. Sci. Lett.* **5**, 320–324 (1969).
10. Roedder, E. in *The Handbook of Stratiform and Stratabound Ore-Deposits* Vol. 2, 67–110 (Elsevier, Amsterdam, 1976).
11. Steiger, R. H. & Jäger, E. *Earth planet. Sci. Lett.* **36**, 359–362 (1977).

Three-dimensional structure of a photosynthetic membrane

Kenneth R. Miller

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, USA

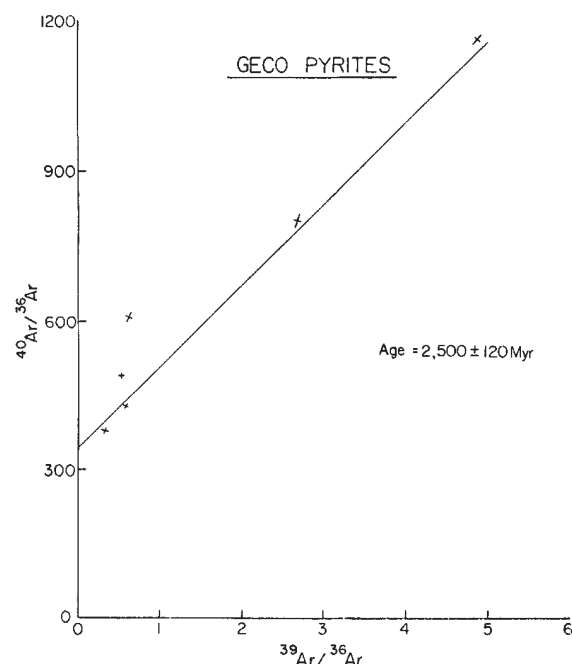


Fig. 2 Isotope correlation plot for Geco pyrites. The least squares best slope through all data points corresponds to an age of $2,490 \pm 200(1\sigma)$ Myr. When the most discrepant point is omitted, the best slope corresponds to an age of $2,500 \pm 120$ Myr. The error bars shown are the principal axes of the error ellipse about each point. The orientation is dependent on the correlation of the $^{40}\text{Ar}/^{36}\text{Ar}$ and $^{39}\text{Ar}/^{36}\text{Ar}$ errors. All error estimates are 1σ .

The photosynthetic membranes in a variety of organisms are involved in the conversion of energy from sunlight into chemically useful forms^{1–3}. *Rhodospseudomonas viridis* is a purple non-sulphur photosynthetic bacterium offering unique advantages for the study of photosynthetic membranes. Its internal photosynthetic membranes form large flat sheets which, as first described by Giesbrecht and Drews⁴, contain subunits organized into a regular repeating pattern. The membranes are ideally suited to the use of Fourier image analysis techniques, and have been studied in two dimensions^{5,6}. We have now determined the three-dimensional structure of photosynthetic membranes of *R. viridis* by electron microscopy. A large central structure protrudes from both surfaces of the membrane, and is surrounded by six smaller centres of mass. Comparison of these data with studies on the polypeptide composition of the membrane suggests a membrane in which a photosynthetic reaction centre is surrounded by light-harvesting bacteriochlorophyll protein complexes.

Membranes were isolated from pressure-disrupted cells as described elsewhere⁷. The membranes were stained with 2% aqueous uranyl acetate on carbon films and examined at tilt angles in an electron microscope ranging from 0° to 61° (Fig. 1). Densitometry and computer processing were carried out on selected micrographs to produce Fourier terms for computing the structure. Fourteen micrographs were used for the analysis, each of which provided as many as 22 individual measurements of amplitude and phase. Many of the micrographs showed regular detail in Fourier transforms extending to four orders (the lattice constant measured from these transforms and from electron diffraction patterns is $100 \text{ \AA}$, corresponding to a centre-to-centre distance for membrane subunits of $115 \text{ \AA}$),

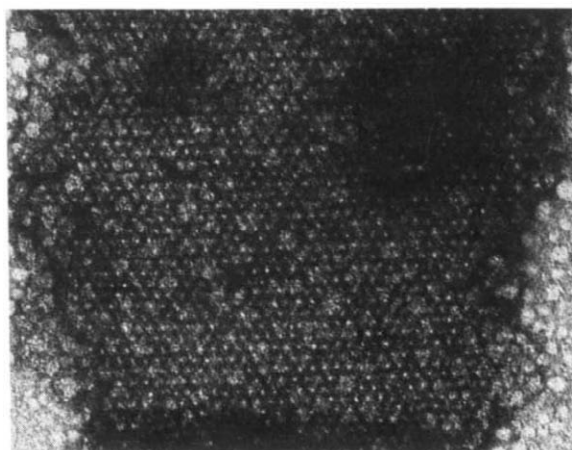


Fig. 1 Isolated photosynthetic membrane from *R. viridis*. The individual subunits can be seen arranged in a hexagonal lattice. The preparation was stained with 2% uranyl acetate. $\times 147,000$.

indicating a resolution of 25 Å, but the final analysis was carried out to three orders for a resolution of 33 Å. Individual micrographs showed projected structure and Fourier terms consistent with the crystallographic space group P6, and the three-dimensional analysis was taken with P6 symmetry, at this level of resolution, applied to the data (see Fig. 2). The average phase residual determined from combining the individual images was 22 degrees, and all Fourier terms were considered in computing phase residuals.

To achieve a proper scaling of amplitude terms in the direction perpendicular to the plane of the membrane, 'edge-on' views of the membrane were also gathered. We used these images in a way similar to that described by Unwin and Zampighi⁸ to calculate the 0, 0 lattice line through the origin of the three-dimensional Fourier transform.

The map calculated by these procedures highlights the stain-excluding structures of the membrane, and is subject to the usual difficulties and distortions associated with negative staining. An appropriate contour value was selected and used to produce an outer surface for the structure of each subunit in the membrane. The complete three-dimensional structure of such a subunit is shown in Fig. 3a.

A large central structure protrudes from each surface of the membrane, and a ring of smaller centres of mass surrounds it. The approximate width of the entire subunit is 100 Å, and the central region is approximately 70 Å thick. The central protrusion dominates the reconstructed image of the membrane; this domination can be appreciated from individual contour sections as well as from the surface map. Figure 3b shows a section through the subunit at the centre of the membrane, with the large central mass region clearly defined. The six

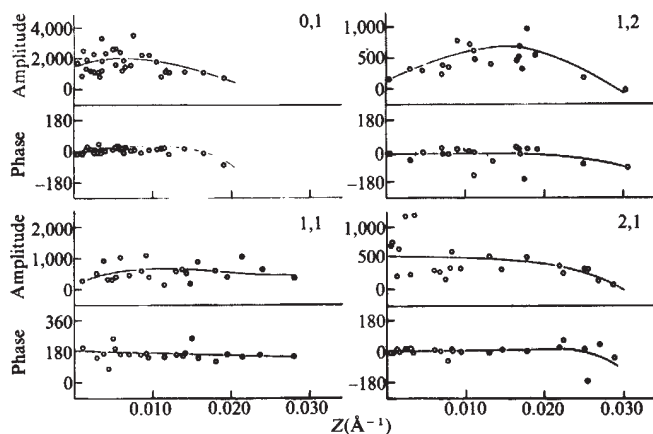


Fig. 2 Phase and amplitude plots of four major lattice lines used for the three-dimensional reconstruction. Values were sampled from these curves for the three-dimensional reconstruction.

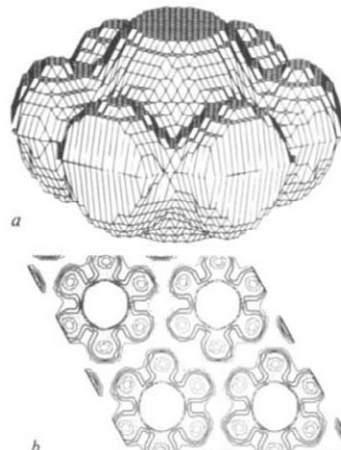


Fig. 3 a, Three-dimensional model of an individual membrane subunit. The surface was generated by applying a contour value to the reconstruction data and generating a surface envelope to fit the contour. A large central structure is seen which protrudes from each surface of the membrane, and six smaller lobes surround the protrusion. b, Four unit cells, arranged as they exist in the membrane, sectioned along a central plane parallel to the membrane sheet. Only the density of the central region can be seen (and not the projected density of the whole structure). In each subunit, the central region is surrounded by the six 'satellite' regions of mass density. Each central region is completely separated from its neighbour by a ring of such satellites.

'satellite' regions are also visible, however, showing that the mass of the subunit is not completely restricted to one main region. The model agrees closely with images obtained from other techniques, including deep-etching and rotary shadowing (Fig. 4).

Several workers have prepared reaction centres from *R. viridis* membranes^{9,10}. X-ray and neutron diffraction studies^{11,12} of similar reaction centres, from *Rhodospseudomonas sphaeroides*, in artificial lipid membranes, suggested an asymmetric mass distribution perpendicular to the membrane plane. The *R. viridis* membrane does not display such strong asymmetry, but note that we have used intact membranes rather than isolated reaction centres.

The *R. viridis* photosynthetic membrane contains light-harvesting components as well as the reaction centre apparatus^{7,13,14}. A recent study in this laboratory revealed seven

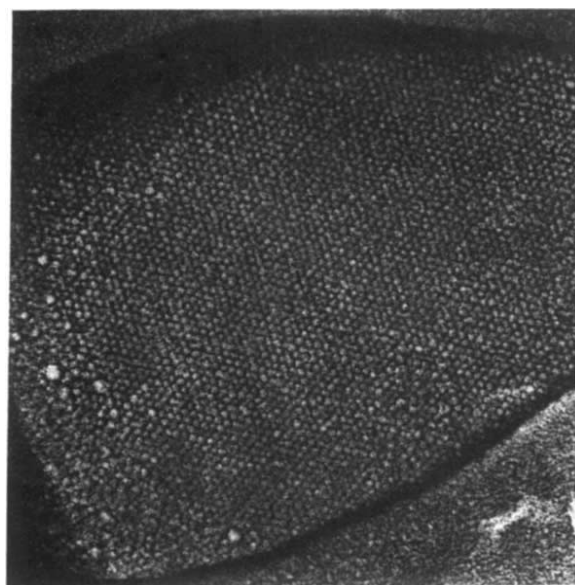


Fig. 4 Outer surface of the *R. viridis* photosynthetic membrane, exposed by deep-etching and rotary shadowing in a freeze-etching device. The lattice is clearly visible and individual subunits are well separated. $\times 100,000$.

polypeptides associated with the intact membrane⁷. Two of the polypeptides (with apparent molecular weights of 11,000 and 16,000) bind bacteriochlorophyll and function in light-harvesting. Of the remaining five, three are associated with the reaction centre itself and two may have a role in electron transport⁷. Interestingly, an analysis of the apparent relative amounts of the seven polypeptides showed that the light-collecting components were present in 6–10 times higher concentration than the other five⁷. It thus seems reasonable to suggest that the 'satellites' of mass around the central structure are related to the two light-collecting components, and that the other five polypeptides (of molecular weights 24,000–44,000) are located in the central structure.

Can the membrane subunits visualized in this study accommodate the seven polypeptides associated with the membrane? We have estimated the volume of each membrane subunit at $\sim 550,000 \text{ \AA}^3$ (the volume of a disk 120 $\text{\AA}$ in diameter and 50 $\text{\AA}$ thick). Using a value of $1.3 \text{ \AA}^3$ per dalton (derived from bacteriorhodopsin¹⁵), we might expect such a volume to accommodate approximately 423,000 daltons. Using the molar

ratios for the seven membrane polypeptides reported by Jacob and Miller⁷, we arrive at a value of 535,000 daltons per reaction complex. Although the molecular weight determination is slightly higher than the available volume, the negative staining used to prepare the samples for electron microscopy may have compressed the sample during the drying process, and could easily account for the slight difference. I therefore suggest that each subunit in the membrane contains a complete set (but only one) of reaction centre, electron transport and light-collecting components.

The programs used for data transformation and image recombination were kindly provided by Drs Linda Amos and Richard Henderson. A number of students and co-workers, including Jules Jacob and Yvonne Alvarez, helped with this project. Special assistance with data processing was provided by David Laidlaw, Tanya Falbel and David Salesin. I especially thank Drs Timothy Baker and David deRosier for the use of electron microscopy and densitometry equipment and for advice at several stages of this project. This work was supported by NIH grant GM-28799.

Received 7 July; accepted 7 September 1982.

1. Arntzen, C. J. *Curr. Topics Bioenergetics* **8**, 111–160 (1978).
2. Miller, K. R. *Scient. Am.* **241**, 4, 102–113 (1979).
3. Niederman, R. A. & Gibson, K. D. in *The Photosynthetic Bacteria* (eds Clayton, R. K. & Sistrom, W. R.) 79–118 (Plenum, New York, 1978).
4. Giesbrecht, P. & Drews, G. *Arch. Mikrobiol.* **54**, 297–330 (1966).
5. Miller, K. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6415–6419 (1979).
6. Wehrli, E. & Kubler, O. in *Electron Microscopy at Molecular Dimensions* (eds Baumeister, W. & Vogell, W.) 48–56 (Springer, Berlin, 1980).

7. Jacob, J. & Miller, K. R. *Archs Biochem. Biophys.* (submitted).
8. Unwin, P. N. T. & Zampighi, G. *Nature* **283**, 545–549 (1980).
9. Thornber, J. P., Cogdell, R., Seftor, R. & Webster, G. *Biochim. biophys. Acta* **593**, 60–75 (1980).
10. Case, G. D., Parson, W. W. & Thornber, J. P. *Biochim. biophys. Acta* **223**, 122–128 (1970).
11. Pachence, J. M., Dutton, P. L. & Blasie, J. K. *Biochim. biophys. Acta* **548**, 348–373 (1979).
12. Pachence, J. M., Dutton, P. L. & Blasie, J. K. *Biochim. biophys. Acta* **635**, 267–283 (1981).
13. Pucheu, N., Kerber, N. L. & Garcia, A. F. *Archs Microbiol.* **101**, 259–272 (1974).
14. Trosper, T. L., Benson, D. L. & Thornber, J. P. *Biochim. biophys. Acta* **460**, 318–331 (1977).
15. Henderson, R. & Unwin, P. N. T. *Nature* **257**, 28–32 (1975).

Growth effects of SO₂ and/or NO₂ on woody plants and grasses during spring and summer

Mary E. Whitmore & Peter H. Freer-Smith

Department of Biological Sciences, University of Lancaster, Lancaster LA1 4YQ, UK

The two most widespread air pollutants in Britain are SO₂ and NO₂ (refs 1, 2). These not only contribute to acidity in rain³, which may seriously affect plant growth, but also cause substantial effects by their direct action as gaseous pollutants. Research into the effects of the gaseous pollutants on grasses has shown that the combination of SO₂ and NO₂ may be particularly toxic to plant growth^{4,5}. Further research in this laboratory showed that *Phleum pratense* L. was more sensitive to SO₂ when growth was slow and it was suggested that exposures during winter may be particularly phytotoxic⁶. Here we present the results of experiments in which serious effects on plant growth were detected even during the summer months. Autumn-sown smooth stalked meadow grass (*Poa pratensis* L.) exposed to SO₂ or SO₂ plus NO₂ showed marked growth reductions during the winter and spring, and although the plants recovered during the summer, flowering was inhibited. Moreover, four broad-leaved tree species showed substantial decreases in growth when exposed to SO₂ plus NO₂ between March and August.

Experiments were done in four large hemispherical glass-houses situated outdoors, in which environmental conditions were close to ambient⁷. Pollutants were added to three of the glasshouses between 09.00 h on Monday and 17.00 h on Friday to give concentrations of 0.1 p.p.m. NO₂, 0.1 p.p.m. SO₂ and 0.1 p.p.m. NO₂ + 0.1 p.p.m. SO₂; weekly mean concentrations were 0.062 p.p.m. (Annual means of 0.06 p.p.m. SO₂ and 0.06 p.p.m. NO₂ may be experienced in several urban areas in Britain^{8–10} where grasses and trees are grown for amenity purposes.) The fourth glasshouse (control) received partially filtered air without added pollutants.

The amenity grass *Poa pratensis* L. cv. Monopoly was exposed to SO₂ and/or NO₂ for 11 months, during which growth was

monitored. Seeds were sown in each treatment on 2 October 1980 and after emergence 15 days later, 110 seedlings were potted into individual 7.5 cm pots containing John Innes no. 2 compost and placed at random within the relevant glasshouse. Samples of 10 plants per treatment were removed for harvesting at intervals until 7 September 1981. Plants were repotted into 10 cm pots in May, after which no further root weight measurements were made. The increases in control plant weight up to mid-May and in shoot weight at this and subsequent harvests are shown as natural logarithms in Fig. 1a; per cent reductions for each pollution treatment are illustrated in Fig. 1b. Between November and February, SO₂ and NO₂ appeared to interact causing greater than additive effects on plant weight; large reductions were evident in the combined gas treatment, while SO₂ alone caused only small reductions, and NO₂ alone promoted growth ($P < 0.05$). After this time, however, the interaction was lost and effects became approximately additive because small reductions developed in the presence of NO₂. During late spring and summer the polluted plants gradually recovered, until by September, shoot weight in NO₂ was similar to that in controls and that in SO₂ and SO₂ + NO₂ exceeded the controls by $\sim 17\%$ ($P < 0.05$). Culm development before flowering was first observed in mid-May and was followed by inflorescence

Table 1 Mean increases in total dry weight (g) of 14 seedlings and 14 cuttings of *Betula pendula* grown in clean air and in air containing 0.05 p.p.m. SO₂ + 0.05 p.p.m. NO₂

	Seedlings	Cuttings
Control		
Mean dry weight $\pm$ s.e.	4.30 $\pm$ 0.376	14.56 $\pm$ 0.553
c.v. (%)	32.7	14.2
SO ₂ + NO ₂		
Mean dry weight $\pm$ s.e.	3.11 $\pm$ 0.367	9.14 $\pm$ 0.373
c.v. (%)	44.2	15.3
Decrease in growth of polluted plants (%)	27.7	37.2
LSD between treatment and control means (%), $P = 0.05$	25.1	9.4

The percentage decrease in growth and the least significant differences (LSD) between the polluted and control means for the seedlings and cuttings are also shown.

development towards the end of the month; Fig. 1c shows mean numbers of culms per plant. SO_2 , whether supplied alone or together with NO_2 , not only delayed stem elongation but also reduced the total number of flower heads to less than half that on controls. The inhibition of flowering may have contributed to the recovery of shoot weight in these treatments by allowing vegetative growth to continue at faster rates than in the controls.

As little is known of the influence of SO_2 and NO_2 mixtures on tree growth, six species were selected for comparison. By using cuttings of only one genotype the variability between replicates can be reduced, and as only a few trees of each species could be accommodated in the fumigation chambers, this meant that small treatment effects would not be masked by plant variability. Therefore, second-year cuttings of *Malus domestica* Borkh. (apple MM 106), *Betula pendula* Roth. (silver birch),

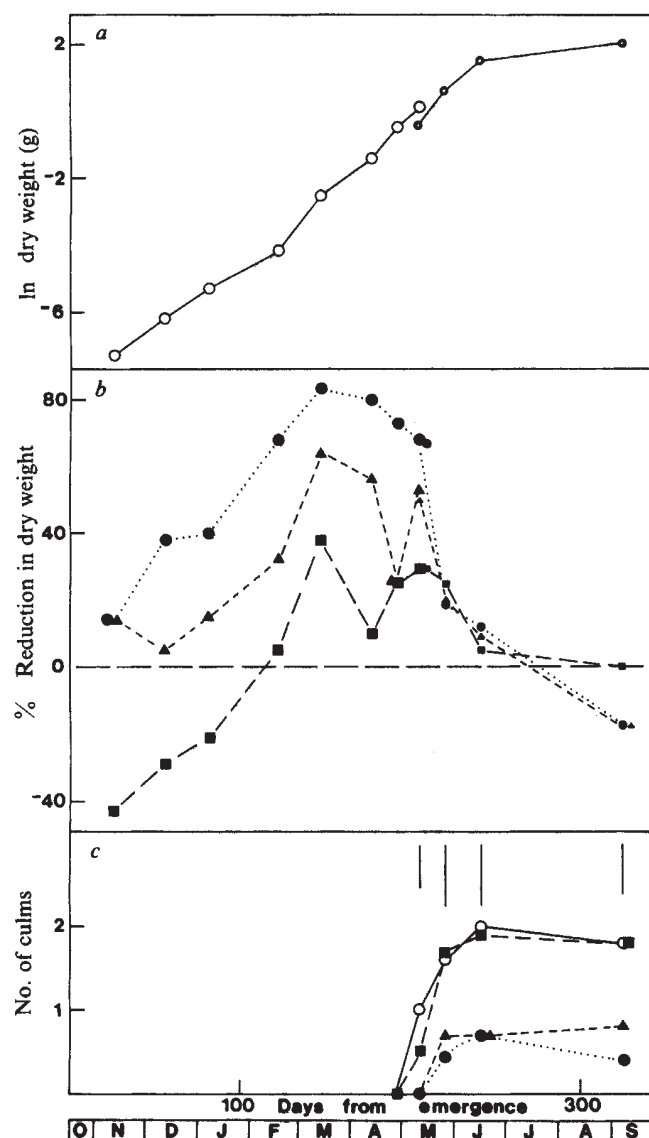


Fig. 1 a, Growth of *Poa pratensis* L. in partially filtered air. Plant weights are shown up to May (large symbols) and shoot weights at this and subsequent dates (small symbols). b, Percentage reductions in dry weight compared with controls after exposure of plants to weekly mean concentrations of 0.062 p.p.m. NO_2 (—■—), 0.062 p.p.m. SO_2 (---▲---) or 0.062 p.p.m. $\text{SO}_2 + 0.062$ p.p.m. NO_2 (····●····). Large symbols represent effects on plant weight and small symbols effects on shoot weight. c, Development of culms: control (—○—), in NO_2 (—■—), and in SO_2 (---▲---) or $\text{SO}_2 + \text{NO}_2$ (····●····). Bars represent LSD, $P = 0.05$.

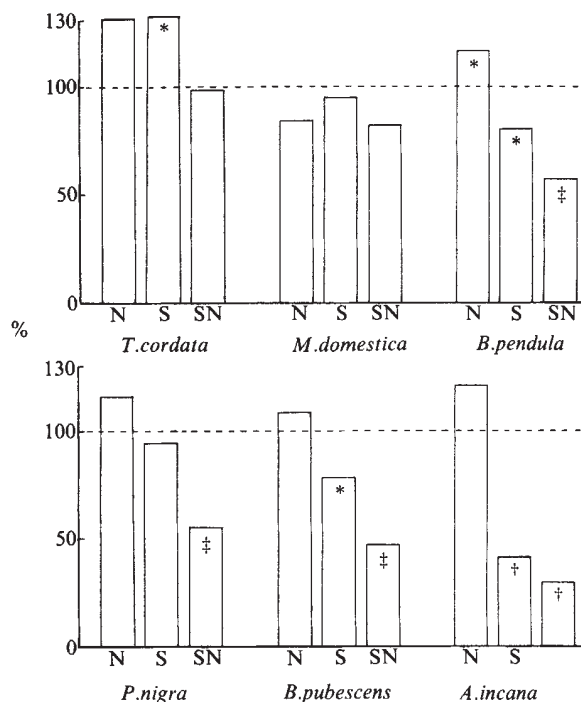


Fig. 2 The mean dry weights of woody plants ($n = 5-8$) exposed to weekly mean concentrations of 0.062 p.p.m. SO_2 (S), NO_2 (N), or $\text{SO}_2 + \text{NO}_2$ (SN) expressed as the percentage of the mean dry weights of controls grown in clean air. Treatment means that were significantly different from control means are marked: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

Betula pubescens Ehrh. (common birch), *Populus nigra* L. (black poplar), and *Alnus incana* L. (grey alder), and seedlings of *Tilia cordata* Mill. (small-leaved lime) were exposed to SO_2 and/or NO_2 from before bud break (23 March) and harvested after 150 days on 19 August 1981. Plants were grown in individual 19 cm Fyba pots containing J.I. no. 2, and were randomly positioned in each of the four glasshouses. The range of responses of the different species to SO_2 and/or NO_2 can be seen in Fig. 2, where the mean dry weights of the fumigated plants are expressed as percentages of the control means. In those species which showed reduced growth in response to pollutants, the combined treatment was the most toxic. There were interactive effects of SO_2 and NO_2 in *T. cordata*, *B. pendula*, *P. nigra* and *B. pubescens*, resulting in greater than additive responses. Those plants which were significantly affected in terms of dry weight also showed significant decreases in stem height and diameter. In some cases visible leaf blemishes were observed on the older leaves of polluted plants, and towards the end of the exposure period accelerated leaf senescence and abscission of the leaves occurred. The early loss of leaves from deciduous trees exposed to small concentrations of SO_2 is well known¹¹, and in the experiment reported here was found to be greater when NO_2 accompanied SO_2 .

To compare the effects of a $\text{SO}_2 + \text{NO}_2$ mixture on seedlings with effects on cuttings (of one genotype), a further experiment was conducted. Seeds of *B. pendula* collected in autumn 1979 were sown the following summer and cuttings from one seedling were bulked up by micropropagation; 14 seedlings and 14 cuttings were grown in two fumigation chambers from 27 May to 14 August 1981. The chambers were kept outside and were rapidly ventilated with charcoal-filtered air. Pollutants were added to one chamber to give an exposure concentration of 0.05 p.p.m. $\text{SO}_2 + 0.05$ p.p.m. NO_2 . Table 1 shows the mean increases in dry weight of the seedlings and cuttings over the 80-day exposure period. The cuttings grew more quickly than the seedlings, but both groups of plants showed decreases in

growth in air containing SO₂ and NO₂. The coefficient of variation (%) of each group is also shown and illustrates the greater variability in the growth of the seedlings. Clearly, a smaller treatment response could have been detected with the clonal plant material than with seedlings.

These data show that growth of deciduous species may be severely decreased by exposure to SO₂ and NO₂ mixtures during the growing season. Sensitivity clearly differed between species and the order of relative sensitivity coincided well with assessments made in the field by examining chronic injury at sites near to pollution sources¹². Exposure to the individual gases caused stimulation of growth in *T. cordata*, *B. pendula* and in the grass *P. pratensis* at some harvests, which suggests that the pollutants provided additional sources of nutrients. Such responses have been reported previously for NO₂ and for SO₂ but are usually associated with conditions in which nutrient supply is limited^{13,14}.

The changing response to pollutants of the grass studied here illustrates some of the difficulties of predicting effects in the field and of interpreting results from an individual harvest. However, recovery of polluted plants during late spring and summer may not be observed in the field if plants are in competition with less sensitive species. An inhibition of flowering when plants are grown in SO₂-containing air may be of possible benefit in turf cultivation, but could have serious implications for commercial seed production.

We thank the NERC for financial support, Professors T. A. Mansfield and F. T. Last for supervision, and Mrs Wendy Tabner for technical assistance.

Received 13 May; accepted 27 August 1982.

1. Fowler, D. & Cape, J. N. in *Effects of Gaseous Pollutants in Agriculture and Horticulture* (eds Unsworth, M. H. & Ormrod, D. P.) (Butterworths, London, 1982).
2. Martin, A. & Barber, F. R. *CEGB Rep. no. MID/SS D/80/0044/N* (1980).
3. Fowler, D. *et al. Nature* **279**, 383–386 (1982).
4. Ashenden, T. W. *Envir. Pollut.* **18**, 249–258 (1979).
5. Ashenden, T. W. & Williams, I. A. D. *Envir. Pollut.* **21**, 131–139 (1980).
6. Davies, T. *Nature* **284**, 483–485 (1980).
7. Ashenden, T. W., Tabner, P. W., Williams, P., Whitmore, M. E. & Mansfield, T. A. *Envir. Pollut.* **B3**, 21–26 (1982).
8. Garnett, A. *Atmos. Envir.* **14**, 787–796 (1980).
9. Annand, W. J. D. & Hudson, A. M. *Atmos. Envir.* **15**, 799–806 (1981).
10. Garnett, A. *Atmos. Envir.* **13**, 845–852 (1979).
11. Garsed, S. G., Farrar, J. F. & Rutter, A. J. *J. appl. Ecol.* **16**, 217–226 (1979).
12. Last, F. T. *Agric. Envir.* (in the press).
13. Cowling, D. W. & Lockyer, D. R. *J. exp. Bot.* **29**, 257–265 (1978).
14. Anderson, L. S. & Mansfield, T. A. *Envir. Pollut.* **20**, 113–121 (1979).

Falcon visual sensitivity to grating contrast

Joy Hirsch

Department of Ophthalmology and Visual Science,
Yale University School of Medicine, New Haven,
Connecticut 06510, USA

The eye of a small falcon, the kestrel, is about half the size of the human eye and, if similar in all other respects, its visual acuity would also be expected to be about half that of the human. Here we report that the cut-off frequency (a measure of visual acuity) is twice the expected value and is the same for kestrel and human eyes. This remarkable visual performance is explained by anatomical features of the bird eye and the spherical pit of the deep fovea acting as the diverging element of a telephoto lens to magnify the image^{1,2}. In the low-spatial frequency range, however, falcon contrast sensitivity is much less than that of humans for stationary patterns.

Experiments were carried out with a 2-year-old male American kestrel, *Falco sparverius*, called Argus, hatched in the wild and acquired at about 1 month old. Stimuli were sinusoidal modulations of a homogeneous luminance field, that is, vertical spatial frequency gratings, electronically generated on the screen of a cathode ray tube (Tektronix 606; P31 phosphor). Mean luminance of the field was ~40 cd m⁻² (calibrated by Tektronix photometer, J16), and was unchanged by modulation. Grating contrast (depth of modulation) was defined as $(L_p - L_t)/2\bar{L}$ where L_p and L_t represent the luminance levels at the peak (maximum) and trough (minimum) of a spatial cycle, and $\bar{L}$ is the average luminance across the screen. Contrast was expressed on a logarithmic scale where contrast in decibels, dB, is equal to $20 \log_{10}(\text{contrast})$. Two conditions were used: (1) stationary gratings with abrupt onset and offset and (2) phase changing gratings that abruptly reversed the sign of modulation every 2 s (0.25 Hz phase reversal). The screen subtended approximately 3 degrees of visual angle on the falcon eye at a distance of 200 cm. A thin vertical line (5 mm wide) bisected the screen into right and left fields. During a trial one side of the bipartite field contained the grating and the other side remained homogeneous at the same average luminance. Stimulus generation and control of the experimental procedure were completely computer-controlled (Digital Equipment Corporation; PDP-11/03).

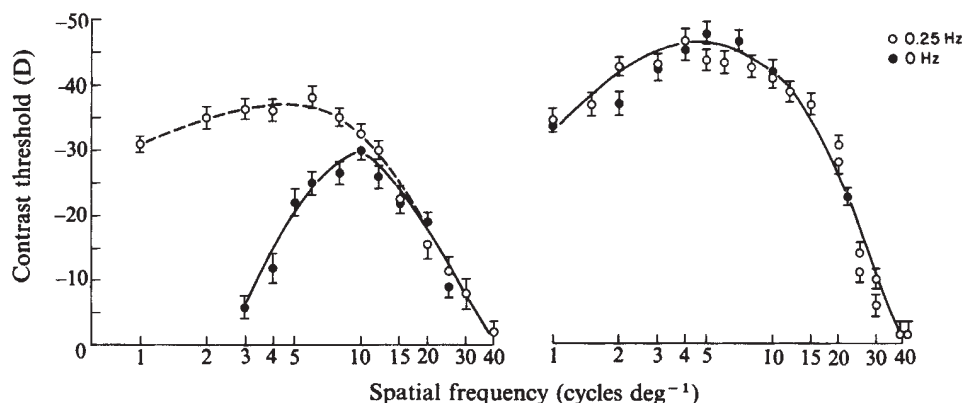
The experimental apparatus consisted of three perches and a food dispenser. The computer was signalled when the kestrel landed on any one of the perches. The perch from which the stimulus was viewed, P₀, signalled that the kestrel was in the observing position, and initiated presentation of the stimulus. Two response perches, P_r and P_l, corresponding to either the right or left bipartite fields, respectively, were located 5 cm in front of and slightly below the stimulus screen, and signalled a response when pressed. The food tray was located directly above the screen and dispensed a small piece of beef heart when signalled by the computer.

The task for the kestrel was to initiate a trial by landing on P₀, decide if the grating was on the right or left side of the field, and fly to either P_r or P_l to indicate his choice. Argus appeared to scrutinize the stimulus field monocularly by turning his head first with one side towards the stimulus field and then the other before making his response flight. This suggests that the nasal (deep) fovea was used for the judgements required in this experiment. (The nasal fovea contains the highest density of photoreceptors and is used for distant viewing¹.) As soon as Argus left P₀ the modulation was automatically removed from the screen to ensure that the response decision had been made at P₀. A food reward immediately followed each correct response. An incorrect response was followed by an auditory signal and the mean luminance was reduced to 0 for 50 s during which a new trial could not occur. Following the 'dark time' the screen returned to the mean luminance level as a ready signal for the next trial. Argus was allowed 100 s of viewing time on P₀ before a trial was aborted and a new stimulus presented. A trial was also stopped if Argus left P₀ and failed to arrive at either P_r or P_l within 3 s.

A two-alternative forced choice method of constant stimuli was used. The stimuli for each session consisted of one spatial frequency presented at four contrast levels each separated by 5 dB. The side of the screen receiving the modulation was randomly chosen, and the order of presentation of each of the stimuli was also randomized. The spatial and temporal stimulus conditions were selected in irregular order across sessions. Each session consisted of 200 trials, and all trials were included in the analysis. As the experimental procedure was automated, much of each session proceeded in the absence of an experimenter. Usually a session lasted 4–5 h. All sessions started in the early evening.

The probability of a correct response was plotted against stimulus contrast and the psychometric function was fit by eye. The contrast level associated with 75% performance was interpolated from the psychometric function to determine contrast

Fig. 1 Contrast threshold (dB) plotted against spatial frequency (cycles deg^{-1}) for falcon (a) and human (b) observers. Closed symbols represent thresholds for stationary gratings (0 Hz), and open symbols represent thresholds for gratings that abruptly reversed phase every 2 s (0.25 Hz). Data from three human observers (L.K., B.M. and J.H.) are combined on the human function. Error bars represent 1 s.d. of threshold estimates repeated for determination of reliability on about one-third of the data points.



threshold. A threshold was obtained only when the per cent of correct responses extended from 50% to ~90% or more. Many contrast thresholds were replicated at 4- and 6-month intervals to confirm stability of the measurements. A catch trial condition was periodically used where no pattern was presented on either side of the screen. Performance did not significantly differ from 50% in such conditions.

Contrast thresholds were similarly determined for three human observers (L.K., B.M. and J.H.) in the same experimental set-up as was used for Argus. Response keys were substituted for perches, and a synthesized voice unit (Votrax, VS-K) was substituted for the beef heart dispenser. The voice unit provided verbal feedback following each trial; otherwise, the software and data analysis for both experiments were identical. Viewing was binocular for the human observers.

Contrast at threshold was plotted against spatial frequency for both stimulus conditions and is shown in Fig. 1 for falcon and human observers. The data are presented so that the ascending direction of the ordinate represents decreasing contrast and, therefore, increasing sensitivity. The sensitivity of the falcon to stationary gratings was dramatically depressed below 10 cycles deg^{-1} relative to the phase changing gratings, suggesting that falcon vision may be highly dependent on temporal cues for discrimination of spatial frequency gratings below 10 cycles deg^{-1} . A similar low frequency fall-off has recently been reported for the contrast sensitivity function of a large eagle, *Aquila audax*³. Human contrast sensitivity functions were similar for both conditions. Absolute levels of peak contrast sensitivity (phase changing gratings) were approximately 0.5 log units lower for the kestrel than for the human. Increased photoreceptor noise, and/or reduced photon capture (possibly due, in part, to the small photoreceptors of the falcon eye) may account for the lower peak sensitivity.

Cut-off frequency for both human and falcon functions was ~40 cycles deg^{-1} . The human spatial cut-off frequency of 40 cycles deg^{-1} observed in this experiment is expected for the luminance level used here⁴. The results for the kestrel are inconsistent with a previous report that kestrel acuity is ~2.6 times higher than that of humans⁵. However, the similar cut-off frequencies for both human and falcon reported here can be explained in terms of the known geometry of the falcon eye and retina.

The falconiform eye is distinguished from other vertebrate eyes by a two-foveal retina^{1,6,7}, closely-packed foveal photoreceptors^{1,2} and superior optics⁸. In general, cut-off frequency of an eye can be estimated from focal length, photoreceptor spacing and pupil diameter^{9,10}. Pupil diameter, measured periodically during the experiment by placing a rule about 1 cm away from the eye while Argus was on the observer perch, was ~2.5–3.0 mm, and thus sufficient to pass the spatial frequencies used in this experiment². In falconiform eyes, focal length, F , is ~0.65 of the axial length, L , and can be estimated from head width, W , where $W = 2L$ ^{1,11}. Head measurements made with

calipers indicate that $W = 28$ mm for Argus. Thus $F = 9.1$ mm, which is about half the human focal length¹⁰, and therefore limits acuity by a factor of half relative to that of humans. However, centre-to-centre spacing of falconiform foveal photoreceptors, d_{cc} , is 2 μm (refs 1, 2), which is two-thirds of the human value and provides a 1.5 factor acuity advantage for the falconiform. Based on focal length and centre-to-centre spacing of photoreceptors, the predicted cut-off frequency for the falcon would be three-quarters of the observed human cut-off frequency or 30 cycles deg^{-1} against an observed cut-off frequency of 40 cycles deg^{-1} . The boost in falconiform spatial frequency cut-off can be accounted for by an image magnification factor m , as given in the equation²

$$\nu = \frac{F}{d_{cc}\sqrt{3} \times 57.3} \times m$$

where ν is the spatial cut-off frequency in cycles deg^{-1} , and F and d_{cc} are as defined above. If m , the foveal magnification factor, is set equal to 1 for humans then the equation gives a magnification factor of 1.33 for the falcon. Magnification factors due to the spherical pit of the deep fovea have been estimated to be ~1.10–1.25 for the eye of a jumping spider (Salticidae)¹² and 1.45 for a human-size falconiform eye¹. The magnification factor of 1.33 reported here is consistent with previous estimates and thus may explain the extraordinarily high visual acuity of the falcon given both the advantage of small photoreceptors and the limitation of small eye size.

I thank the following for contributions: William Miller, Ron Hylton, Lynn Kohlmeier, Ben Miller, the Wildlife Unit (Department of Environmental Protection, Connecticut), and the US Fish and Wildlife Service. This work was authorized by Federal Scientific Collectors Permit PRT 2-889 BO and State of Connecticut Wildlife permit granted under Section 26-60 (Connecticut Statutes), and was supported in part by grants from Research to Prevent Blindness, NEI grants EY 00785 and EY 00167-01, and the Connecticut Lions Eye Research Foundation Association.

Received 12 January; accepted 10 September 1982.

1. Snyder, A. & Miller, W. *Nature* **275**, 127–129 (1978).
2. Miller, W. H. *Handbook of Sensory Physiology* Vol. 7/6A, 114–122 (Springer, Berlin, 1979).
3. Raymond, L. & Wolfe, J. *Vision Res.* **21**, 263–271 (1981).
4. Patel, A. S. *J. opt. Soc. Am.* **56**, 689–694 (1966).
5. Fox, R., Lehmkuhle, S. W. & Westendorf, D. *Science* **192**, 263–265 (1976).
6. Fite, K. V. *Brain Behav. Evol.* **12**, 97–155 (1975).
7. Walls, G. L. *The Vertebrate Eye* (Cranbrook Institute of Science, Bloomfield Hills, Michigan, 1972).
8. Shlaer, R. *Science* **276**, 920–922 (1972).
9. Barlow, H. B. *Photophysiology* Vol. 2, 162–202 (Academic, New York, 1964).
10. Westheimer, G. *Handbook of Sensory Physiology* Vol. 7/2 (Springer, Berlin, 1972).
11. Pumphrey, R. J. in *Biology and Comparative Physiology of Birds* (ed. Marshall, A. J.) (Academic, New York, 1961).
12. Williams, D. S. & McIntyre, P. *Nature* **288**, 578–580 (1980).

Gigaseal patch clamp recordings from outer segments of intact retinal rods

P. B. Detwiler, J. D. Conner & R. D. Bodoia

University of Washington, School of Medicine,
Department of Physiology and Biophysics, Seattle,
Washington 98195, USA

The hyperpolarizing light response of vertebrate photoreceptors depends on cation-selective ionic channels in the cell membrane, which open in darkness and close in light. Little is known about these channels even though they are crucial elements in the transduction of light into a change in receptor potential. Here we report our attempts to study the light-sensitive channel using a patch pipette technique for recording ionic currents from a small area of outer segment membrane. The electrode was sealed against the membrane with a sealing resistance higher than a gigohm, permitting good resolution of membrane currents^{1,2}. Working at low external calcium concentrations this allowed us to observe in response to light small outward currents (the difference currents observed when the light-sensitive channel closes, thus switching-off an inward dark current), which reversed polarity with membrane depolarization. We were unable to recognize discrete current steps that might represent single channel openings and closings modulated by light. However, the light-induced outward current was accompanied by a decrease in membrane current noise. Analysis showed that the elementary event in 0.1 mM external calcium is ~ 12 fA. If it is assumed that the elementary event is due to the closure of a single light-sensitive channel and that the potentiating effect of low external calcium on the photocurrent is due solely to an increase in single-channel current, one can calculate that in normal calcium the value of the elementary event is 2 fA, corresponding to a single channel conductance of 50 fS. A conductance of this size is consistent with ion permeation via either a mobile carrier or a pore.

Experiments were performed on the dark-adapted retina of the nocturnal lizard, *Gekko gekko*, which was chosen after unsuccessful attempts to obtain stable patch recordings from other types of photoreceptors. The recording method is shown in Fig. 1a. A piece of retina from approximately one-fifth of the eye was isolated and placed in a perfusion chamber with the photoreceptor outer segments oriented parallel to the stage of an inverted microscope. Under visual observation through an IR image converter, the recording pipette was introduced into the chamber from the side and pressed against either the tip or, less frequently, the distal side of the outer segment of single intact rods. For $\sim 60\%$ of the time, brief suction applied to the pipette caused its resistance to increase suddenly from ~ 4 M Ω to 1–50 G Ω , as has been shown with other tissues^{1,2}. Seal resistances in the gigohm range provide an order of magnitude improvement in current resolution over the recordings obtained using the rod 'sucking' technique^{3,4}, and allow us to apply voltage steps to the patch without penetrating the cell with microelectrodes.

Over the course of these experiments, gigaseal recordings were obtained from 202 rods, and 49 of these responded to light with an outward change in membrane current. The reason for the low percentage of responding cells is not known. The membrane at the tip of the outer segment, which is shed during receptor renewal, might be abnormally fragile or not always functional. It is also possible that the low yield of responding cells is due either to damage sustained in preparing the isolated retina or to breakage of the outer segment membrane when suction was applied in attempting to form a gigaseal. The incidence of membrane breakage was highest in low Ca^{2+} solu-

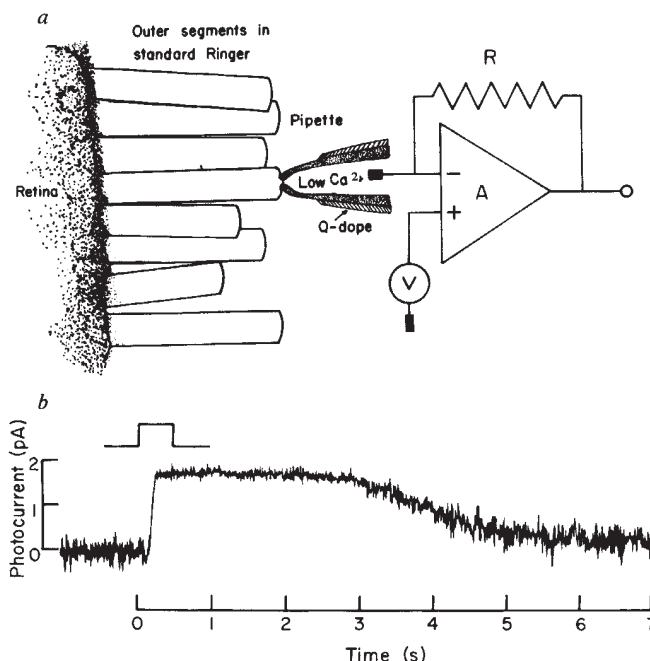


Fig. 1 a, Recording method. The isolated retina was anchored in a chamber through which flowed a continuous stream of oxygenated and chilled ($\sim 18^\circ\text{C}$) Ringer's solution. A fire-polished glass pipette insulated with polystyrene Q-dope (GC Electronics) and filled with low calcium Ringer's solution, was pressed against the tip of a suitable rod outer segment. Gentle suction was briefly applied to the inside of the pipette until the seal resistance abruptly increased to >1 G Ω . Ag/AgCl pellet electrodes were used in the pipette and bath. Current flowing across the patch of membrane at the pipette tip was measured with a virtual ground circuit using a 10 G Ω feedback resistor (R). The potential inside the pipette was controlled by a variable voltage source (V). The output of the amplifier (A) was low-pass filtered at 100 Hz or 160 Hz (8 pole), amplified further and recorded on an FM tape recorder. For all experiments, the Ringer's solution contained (mM): NaCl, 159; KCl, 3.3; CaCl_2 , 1.0; MgSO_4 , 1.0; dextrose, 10; HEPES, 2.8 (neutralized with NaOH to pH 7.8). The pipette filling solution was the same except CaCl_2 was 0.1 mM. b, Light response of a patch of rod outer segment. The trace shows the recording of membrane current (outward current positive) before and after a 500-ms flash of white light (photocell trace on top); bandwidth d.c. to 100 Hz. Stimulus intensity was 6.25×10^{-10} erg μm^{-2} flash $^{-1}$. Seal resistance 1 G Ω .

tions, which set a practical limit to the amount by which the external calcium concentration could be reduced.

Figure 1b shows the response of a gekko rod to a moderately bright flash of white light. The outward photocurrent rose rapidly to a peak amplitude which persisted for a few seconds and then declined slowly. The size of the photocurrent produced by saturating light intensities varied from cell to cell over about a tenfold range, with a mean value of 1.6 pA. For the cells having the largest photocurrents, it was possible to see that a protrusion of outer segment membrane had been drawn into the tip of the patch pipette. Since recordings of this type differed from the others only in the size of their light response, we attributed the variations in photocurrent amplitudes to differences in the area of membrane in the patch pipette.

To establish that the observed photocurrents were due to changes in the conductance of the membrane under the recording pipette, we examined the influence of the patch potential on the light response. The potential across the membrane patch was changed by applying the appropriate voltage to the external surface of the membrane patch via the pipette. The inside of the pipette was made negative for depolarization and positive for hyperpolarization. The outward photocurrent observed at resting potential increased in amplitude with hyperpolarization,

decreased with depolarization, and appeared as an inward current at potentials displaced by more than approximately +40 mV from the resting potential. Detailed analysis of these experiments is complicated by the fact that the intracellular potential changes during the light response, as the whole outer segment was illuminated. Nonetheless, the results provide evidence that the current response is due to a light-induced conductance change of the membrane patch with a reversal potential of about 0 mV.

The current recorded from a patch of outer segment membrane fluctuated in darkness in an apparently random manner. The peak-to-peak noise in the quietest recordings was 0.1–0.2 pA and contained no recognizable discrete current events that could be considered the opening or closing of single light-sensitive channels. In all cells, steady illumination reduced the current fluctuations. For example, in the experiment of Fig. 2,

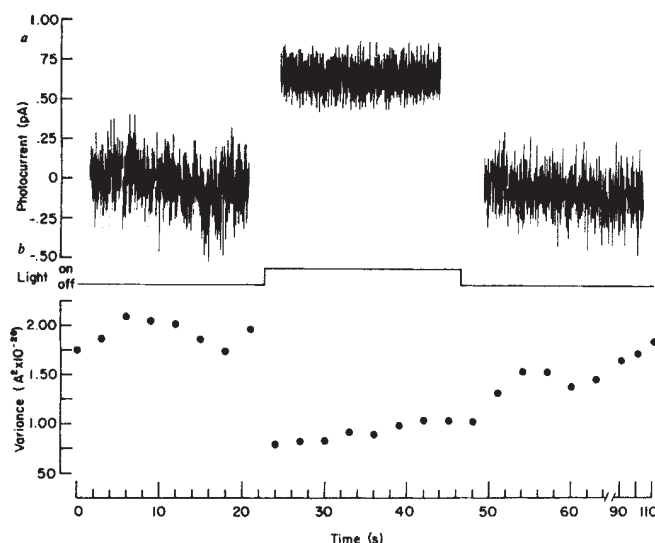


Fig. 2. *a*, Recordings from an outer segment patch in darkness and during steady illumination. The traces shown are representative 2-s long samples of the high gain current record before, during and after steady illumination. The steady outward photocurrent produced by illumination is indicated by the vertical displacement of the trace obtained in light. The output of the light monitor is shown on the middle trace and applies to both parts *a* and *b*. *b*, Graph of the current variance versus time. The continuous current trace was divided into successive 2-s long segments. A laboratory computer (DEC LSI 11/23) was used to digitize signals to form 1,024 points at 2-ms intervals, and to calculate the variance within each 2-s long segment¹⁴. The dark values in the text are the averages of a larger number of values than are shown in the graph. The return of noise following illumination was initially sluggish, approximating the initial dark level 45–60 s after the return to darkness (note break in the time axis). Bandwidth d.c. to 160 Hz; stimulus intensity 326 photons $\mu\text{m}^{-2} \text{s}^{-1}$ at 520 nm; seal resistance 3 G Ω .

the current fluctuated over a range of 0.5 pA (peak-in-peak) in darkness with a mean variance of $1.93 \times 10^{-26} \text{ A}^2$. Steady illumination with a moderate light then produced a maintained outward photocurrent of 0.67 pA, and the mean current variance was reduced to $0.98 \times 10^{-26} \text{ A}^2$. When the light was turned off, the outward photocurrent declined and the noise recovered slowly over the following 60 s to a final mean value of $1.69 \times 10^{-26} \text{ A}^2$. The change in variance caused by steady light was calculated by subtracting the grand average of the variances during light from the grand average of the variances measured during the two dark periods. Based on eight cells for which it was possible to obtain sufficiently long and stable recordings during the dark–light–dark sequence, the average value of the reduction of noise by steady light was $1.9 \times 10^{-26} \text{ pA}^2$. The

approximate magnitude of the elementary events underlying the light-suppressed noise was estimated by dividing the change in current variance by the change in mean current⁵. This calculated unit event size did not depend on the intensity of steady illumination, nor did it correlate with the magnitude of the saturating light response produced by the cell. In the eight cells referred to above, the amplitude of the estimated unit event ranged from 4 to 21 fA, with a mean value of 12 fA.

Our results do not allow us to decide what the unit event represents. It could be the change in membrane current due to the closure of a single light-sensitive channel, or the spontaneous release of a packet of transmitter substance resulting in the fairly synchronous closure of a number of channels. If we assume that the unit event we have measured is due to the closure of a single light-sensitive channel, we can make several inferences about the properties of the channel. The recordings described here were obtained in an external medium containing 1/10th of the normal calcium concentration. Previous measurements in rods have shown that lowering the external calcium concentration increases the light-sensitive current^{6–9}. Decreasing the external calcium concentration from 1 to 0.1 mM is associated with an approximate fivefold increase in photocurrent⁹. The mechanism of this effect is unknown; however, it could be that low calcium concentrations increase the single channel conductance and/or the number of functioning light-sensitive channels. Preliminary experiments have not allowed us to choose between these alternatives. It is interesting, however, that if external calcium influenced the photocurrent solely through an action on the conductance of light-sensitive channels, then the estimated unitary event in normal calcium would drop to 2 fA. This value agrees remarkably well with the shot event size calculated on more theoretical grounds for toad rods¹⁰. With a unit event of this size, a 1 pA single-photon response would require the simultaneous closure of ~500 channels. Furthermore, if the maximum photocurrent produced by the outer segment is between 20 and 30 pA (ref. 4) than the total number of light-sensitive channels would range over 10,000–15,000 per outer segment, giving a channel density of 10–15 μm^{-2} .

The experiments that measured the reversal potential of the light response indicate that the driving force on the light-sensitive channel is ~40 mV. A unit event of 2 fA would then correspond to a single channel conductance of 50 fS. This is ~140 times smaller than the conductance of a single electrically excitable sodium channel¹¹. The flux through a channel this size would be 1.25×10^4 sodium ions per second, which is of the same order as that for a shuttle-type mobile carrier like valinomycin^{12,13}. It has been reported⁹ that in 0 Ca-EGTA the photocurrent increases approximately 20-fold. If we retain the assumption that external calcium influences solely the single-channel current then the flux through the channel could be as large as 2.5×10^5 ions per s. This rate of ion permeation is intermediate between what could be accomplished by a carrier and what is clearly a pore. The argument for a mobile carrier would be strengthened if the unit event we have measured involved a number of permeation sites. Our results are thus compatible with either a carrier or a pore being responsible for ion movement through light-sensitive channels in the rod outer segment.

We thank Drs W. Almers, D. Baylor, M. Binder, B. Hille and R. Ruff for their help. This work was supported by grant EY02048 from the National Eye Institute, USPHS.

Received 12 July; accepted 16 August 1982.

1. Neher, E., Sakmann, B. & Steinbach, J. H. *Pflügers Arch. ges. Physiol.* **375**, 219–228 (1978).
2. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. S. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
3. Yau, K.-W., Lamb, T. D. & Baylor, D. A. *Nature* **269**, 78–80 (1977).
4. Baylor, D. A., Lamb, T. D. & Yau, K.-W. *J. Physiol., Lond.* **288**, 589–611 (1979).
5. Katz, B. & Miledi, R. *J. Physiol., Lond.* **224**, 665–699 (1972).
6. Yoshikami, S. & Hagins, W. A. *Biochemistry and Physiology of Visual Pigments* (ed. Langer, H.) 245–255 (Springer, Berlin, 1973).
7. Brown, J. E. & Pinto, L. H. *J. Physiol., Lond.* **236**, 575–591 (1974).

8. Lipton, S. A., Ostroy, S. E. & Dowling, J. E. *J. gen. Physiol.* **70**, 747–770 (1977).
9. Yau, K. W., McNaughton, P. A. & Hodgkin, A. L. *Nature* **292**, 502–505 (1981).
10. Baylor, D. A., Mathews, G. & Yau, K.-W. *J. Physiol., Lond.* **309**, 591–621 (1980).
11. Neher, E. & Stevens, C. F. A. *Rev. Biophys. Bioengng* **6**, 345–381 (1977).
12. Luger, P. *Science* **178**, 24–30 (1972).
13. Armstrong, C. M. Q. *Rev. Biophys.* **7**, 179–210 (1975).
14. Lamb, T. D. & Simon, E. J. *J. Physiol., Lond.* **263**, 257–286 (1976).

Cholecystokinin activation of single-channel currents is mediated by internal messenger in pancreatic acinar cells

Y. Maruyama & O. H. Petersen*

The Physiological Laboratory, University of Liverpool, PO Box 147, Brownlow Hill, Liverpool L69 3BX, UK

Cholecystokinin (CCK) is one of several active peptides that have been isolated from both the gut¹ and the brain². CCK evokes membrane depolarization and conductance increase in both pancreatic acinar cells^{3,4} and pyramidal neurones of hippocampus⁵. In the acinar cells, activation of cholinergic muscarinic and two different peptidergic receptor sites (for CCK and bombesin) result in the same type of conductance increase⁶. The application of intracellular Ca mimics the action of the agonists⁷ and Ca has been proposed as an intracellular mediator for the membrane conductance increase⁸. A cation channel activated by internal Ca has been directly demonstrated in single-channel current recording experiments on isolated patches of plasma membrane from the basolateral surface of pancreatic acini⁹. We now demonstrate that CCK and acetylcholine (ACh) can activate these inward current channels indirectly. Single-channel currents were observed in electrically isolated membrane patches *in situ* when micropipette application of CCK or ACh was made outside the patch area. Single-channel currents from the same membrane patches were subsequently recorded after they had been excised (inside-out) and identical values for the single-channel conductance were obtained (~35 pS). No effect of CCK was obtained in the presence of the specific receptor antagonist dibutylryl cyclic GMP^{10–12} although in all such cases single-channel currents were subsequently recorded from the excised patch (Ca-activated). These results demonstrate directly that CCK and ACh control ion channel opening via an intracellular messenger (Ca).

We used collagenase-isolated mouse pancreatic acini prepared as described previously^{13,14}. Single-channel current recordings from the basolateral plasma membrane were made *in situ* or in excised membrane patches (Fig. 1) using the techniques described by Neher and collaborators^{15,16}. The acini were stimulated by local micropipette application of ACh or cholecystokinin-octapeptide (CCK₈; Cambridge Research Biochemicals, CCK_{26–33} octapeptide amide, sulphated) in the presence or absence of the specific CCK antagonist, dibutylryl cyclic GMP (Sigma)^{10–12}.

The experiments always began by recording from patches *in situ* as shown in Fig. 1a. Good seals between pipette and plasma membrane (> 20 G Ω) were obtained in patches from 25 batches of mouse pancreatic acini. In the absence of agonist the patches were, in most experiments, completely silent (Fig. 2a). Spontaneous unitary currents were only observed in two patches; these were not studied further. Patches were sometimes monitored for long periods (up to 20 min) without stimulation. The initially silent patches did not subsequently develop spontaneous activity. In experiments designed to investigate the effect of CCK, this agonist was delivered by diffusion of the hormone from the tip of a micropipette brought close to the acinar unit from which high resolution current recording

was made (Fig. 1a). Because the CCK-containing pipette was moved gradually until the tip was at a distance of about 20 μ m from the site of the electrically isolated patch (an inverted microscope with $\times 500$ magnification was used), it was impossible to obtain very accurate values for the latency of activation of the single-channel currents evoked (Fig. 2b–e). The activity normally appeared 20–40 s after the pipette had reached its final position. In six experiments on six different membrane patches, CCK stimulation resulted in the appearance of unitary current steps of the type shown in Fig. 2b–g. The recordings were very stable and currents at many potential levels could be monitored in each individual experiment. The linear single-channel current–voltage (*I*–*V*) relationship (see Fig. 3a) gave a 35-pS single-channel conductance. After a suitable amount of data had been recorded at various potential levels, the patch pipette was withdrawn from the acinus and the tip passed through the bath solution–air interface once or twice to form an inside-out membrane patch¹⁶ (Fig. 1b). High resolution current recording was continued and unitary current steps were always observed in this condition (Fig. 2h). Again, currents were recorded over a wide range of membrane potentials. The current–voltage relationships shown in Fig. 3a make clear that the single-channel conductance obtained in the excised patches is virtually the same as *in situ*. In the isolated patches, reversal of current polarity always occurred at zero pipette potential (symmetrical solutions) where *in situ*, reversal occurred at a pipette potential of –25 mV (Fig. 3a). A separate series of four experiments on excised patches in which normal ionic gradients were present (physiological saline with ‘extracellular’ composition in pipette and ‘intracellular’ fluid in bath) was also carried out. In these experiments, in which the transmembrane ionic gradients correspond to the *in situ* recording situation, the usual type of unitary current activity was seen (Fig. 2i, j) and *I*–*V* relationships were obtained (Fig. 3b). The single-channel conductance was the same as in the other experiments, with current

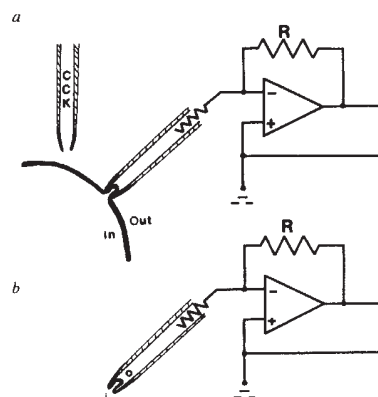


Fig. 1 Schematic outline of experimental system. *a*, Recording from *in situ* membrane patch. Fabrication of patch pipettes and recording system as previously described by Neher and collaborators^{15,16}. In these experiments the recording pipette was always filled with the bath solution, which had the following composition (mM): 140 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.13 MgCl₂, 10 glucose and 10 HEPES. The solution was titrated to pH 7.3 by NaOH. No agonist was present in the pipette solution. The pipette labelled CCK contained the octapeptide in a concentration of 5 μ M dissolved in 0.9% NaCl acidified to a pH of 4.0 by HCl. No currents were applied to this pipette. It has previously been shown that glass micropipettes filled with the CCK analogue caerulein will cause substantial depolarization of mouse pancreatic acinar cells (microelectrode experiments) when the tip is placed close to the outside of an acinus²⁴. Similar experiences have been gained with CCK pipettes. In this work the spontaneous diffusion of CCK from the tip of a micropipette was therefore used as the means of stimulation. In experiments with ACh stimulation the pipette was filled with 2 M ACh–Cl. A braking current of 20 nA was used. During stimulation an ejecting current of 500–1,000 nA was applied. *b*, After the *in situ* recording the patch-clamp pipette was withdrawn from the acinus under investigation and the tip passed through the bath fluid–air interface to obtain an excised inside-out membrane patch¹⁶. In these experiments single-channel recordings were made in symmetrical saline solutions. In other experiments the bath fluid was changed to one having an intracellular composition (mM) of: 145 KCl, 10 NaCl, 1.13 MgCl₂, 10 glucose, 10 HEPES and 10 μ M CaCl₂ with pH of 7.3. This enabled single-channel currents from excised patches to be recorded in conditions with normal transmembrane ionic gradients.

* To whom correspondence should be addressed.

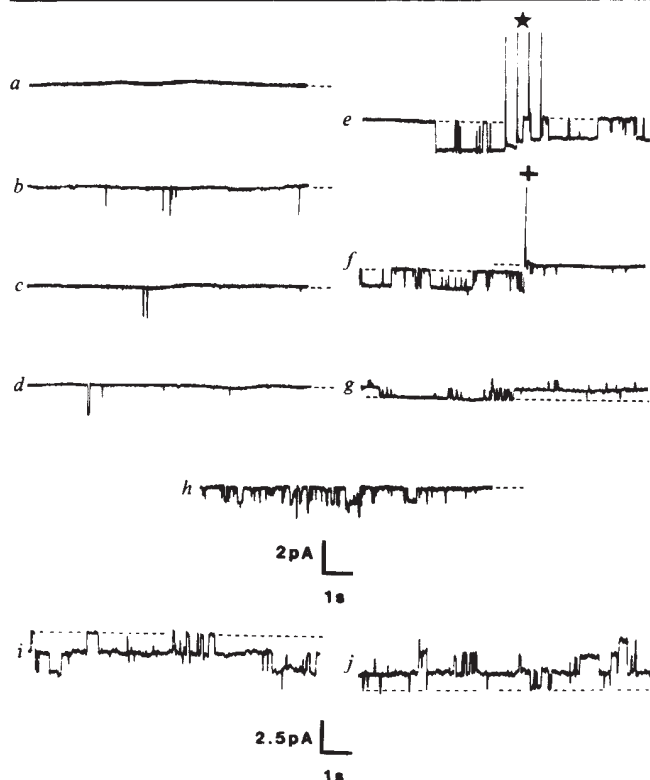


Fig. 2 The effect of CCK application. *a-h*, Consecutive traces from same membrane patch. *a*, Recording situation as described in Fig. 1*a* legend, but with the CCK pipette far away from the acinus under investigation. Pipette potential +40 mV. *b-d*, Three continuous traces obtained 20, 30 and 40 s, respectively, after the tip of the CCK pipette had been brought close to the patch pipette (recording situation as shown in Fig. 1*a*). Pipette potential +40 mV. *e*, 50 s after start of CCK stimulation. At the asterisk the potential of the patch pipette was changed (in steps of 5 mV) from +40 to +20 mV. *f*, Pipette potential +20 mV. At the plus sign the potential was changed to 0. *g*, Pipette potential -40 mV. *h*, Currents from the same patch after excision (inside-out) (recording situation as shown in Fig. 1*b*). Symmetrical extracellular saline solutions. Pipette potential +50 mV. *i, j* show currents recorded from another excised (inside-out) membrane patch with intracellular solution (high K, low Na) in the bath and the normal extracellular solution in the pipette. Pipette potential +60 mV in *i* and -60 mV in *j*. Dashed lines represent the current level when all channels are closed. Downward deflections represent the current from extracellular to intracellular side of membrane.

reversal occurring at a pipette potential of -5 mV (Fig. 3*b*). The difference between this reversal potential and the reversal potential obtained in the *in situ* recording situation (-25 mV) indicates the existence of a 20 mV inside negativity in the intact acini during CCK stimulation. This corresponds reasonably well to values obtained with microelectrodes³.

The effect of CCK application was also investigated in a series of experiments in which a specific CCK antagonist (dibutylryl cyclic GMP)¹⁰⁻¹² was present in the bath solution. In none of the six experiments carried out did CCK stimulation evoke any unitary current steps (Fig. 4*a, b*) although the usual type of currents were readily seen after excision of the patch and exposure of the physiological inside of the membrane to the high Ca concentration of the bath fluid (Fig. 4*c, d*). In one experiment, CCK stimulation for 4 min in the presence of antagonist did not produce any unitary current steps, but when the patch was subsequently excised the usual activity appeared. The effective CCK concentration surrounding the acinar membrane is, of course, not known in this type of experiment. The result, showing that 1.5 mM dibutylryl cyclic GMP can completely abolish the effect of CCK, indicates, however, that the CCK concentration is unlikely to have been higher than 10^{-9} M (ref. 10).

The effect of ACh stimulation was investigated in experiments with ionophoretic agonist application. In these experiments the ACh pipette could be brought close to the acinus without stimulating the cells by applying a braking (retaining)

current. Effects of ejecting ACh from the pipette by ionophoresis were observed after latencies of about 10-40 s. The evoked unitary currents were indistinguishable from those evoked by CCK and the *I-V* relationships were virtually identical (Fig. 3*c*). Subsequent current recordings from an excised patch gave the usual pattern of unitary current steps with the same single-channel conductance (Fig. 3*c*).

In three experiments on excised inside-out patches (extracellular solution on both sides), removal of Ca from the bath (nominally Ca-free solution with 1 mM EGTA) rapidly abolished unitary activity (within 10-20 s), confirming our previous finding on rat pancreas⁹. In the mouse the effect of Ca removal was rapidly (10-20 s) and virtually completely reversible on Ca readmission (300 μ M). In the excised patches the apparent number of channels varied between 2 and 10 with 3 or 4 channels per patch representing a common pattern in these experiments (Fig. 4).

This study of the action of CCK and ACh at the single-channel level provides direct evidence for the hypothesis previously advanced that in several gland cells, hormones and

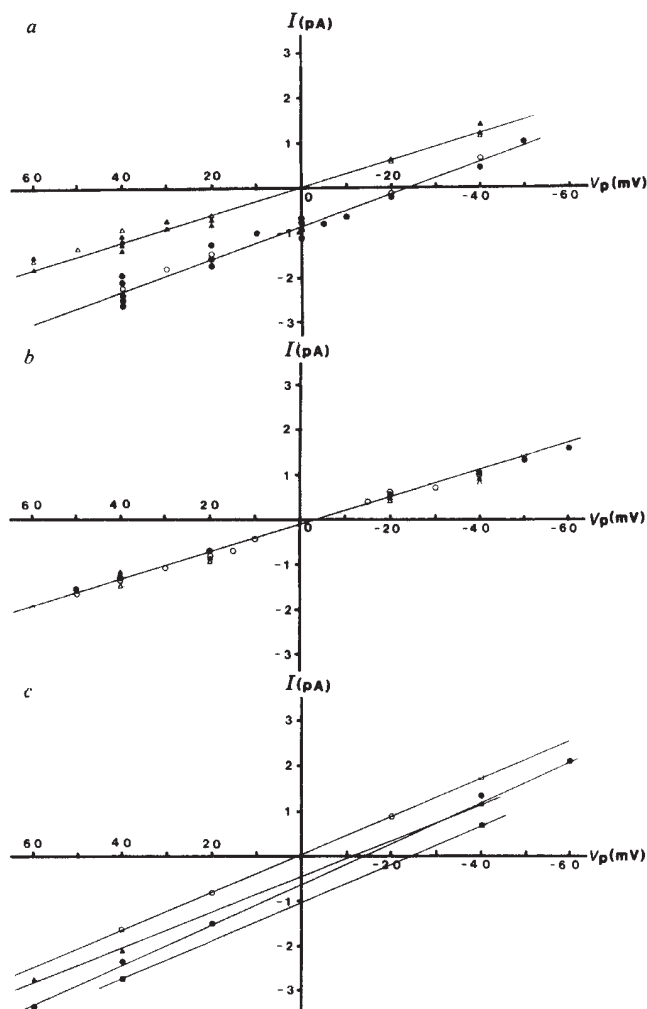


Fig. 3 Single-channel current-voltage relationships. *a*, CCK experiments. Circles represent the data from the six experiments in the *in situ* recording situation. The reversal potential corresponds to a pipette potential of about -25 mV. After excision of the patches (successful in five of the six experiments) the current-voltage relation is as represented by the triangles (symmetrical extracellular solution). The reversal potential corresponds to a pipette potential of 0. The open circles and triangles represent data from the experiment shown in Fig. 2*a-h*. *b*, Data from four excised patches (inside-out) with asymmetrical solutions. Extracellular solution in pipette and intracellular solution in bath. Reversal potential corresponds to a pipette potential of about -5 mV. *c*, ACh experiments. Filled symbols represent three individual experiments where recordings were made *in situ*. Reversal potential corresponds to a pipette potential of about -20 mV. The open circles represent data from the same experiment as the closed circles, but after excision of patch. Symmetrical saline solutions were used and the reversal potential corresponds to a pipette potential of 0.

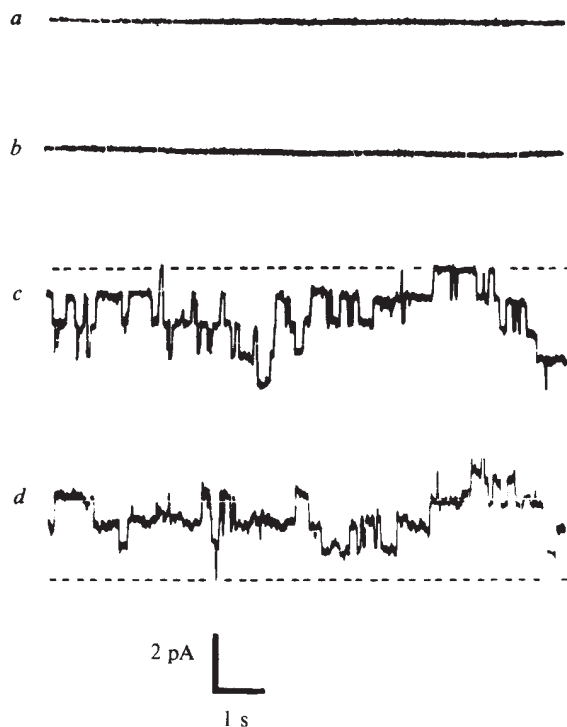


Fig. 4 The effect of CCK in the presence of 1.5 mM dibutyl cyclic GMP in the bathing fluid. *a-d*, Consecutive traces from same patch. Pipette potential +40 mV in *a*, *b* and *c*, but -40 mV in *d*. *a*, *In situ* recording situation as shown in Fig. 1*a*, but with the CCK pipette far away from the acinus. *b*, 100 s after tip of CCK pipette has been brought close to the patch pipette as shown in Fig. 1*a*. *c*, *d*, Same patch, but after excision to expose physiological inside of membrane to extracellular bath solution having a high Ca concentration. Dashed lines represent current level when all channels are closed.

neurotransmitters control membrane permeability indirectly, via an intracellular messenger^{8,17}. CCK-binding studies on pancreatic acini have revealed the existence of about 10,000 receptor sites per cell¹². If our patches have a membrane area of about 10 μm^2 (ref. 16), we estimate that there are 100–600 channels per cell (assuming 2–10 channels per patch and one acinar cell having 600 μm^2 plasma membrane¹⁸). The ion transport pathway involved, a Ca-activated inward current channel, has only recently been described in cultured cardiac cells¹⁹, neuroblastoma cells²⁰ and pancreatic acinar cells⁹. The experiments described here indicate that at least in the pancreatic acinar cells a hormone and a neurotransmitter can control the activation of this channel. Since CCK and ACh have been shown here to activate channels in an area (the patch area) to which these stimulants had no access, the existence of an internal messenger is postulated. Internal Ca can activate the channels and is an obvious candidate.

The mechanism for control of ion transport identified here at the single-channel level, may be the main controlling step for trans-acinar sodium and fluid transport (secretion)^{17,21}. In the toad urinary bladder, current noise measurements by Lindemann and collaborators^{22,23} indicate that the main hormonal (aldosterone and oxytocin) control of transepithelial Na transport (absorption) is exerted at the Na entry step by determining the number of open channels in the luminal membrane. In the pancreas, we calculate that the channels activated by CCK or ACh can account for an inward current, in the whole of an acinar unit, of up to 50 nA at a membrane potential of -30 mV, assuming an average patch area of 10 μm^2 (ref. 16), an average channel density of three per patch—the channels being open half the time⁹, and using known values for the membrane surface of acinar cells and the number of cells in an electrical unit (~ 500)²¹. This maximal current is more than sufficient to account for the macroscopic currents actually measured in experiments with microelectrodes in which 1 μM ACh was

shown to evoke an inward current of about 4 nA, accounting quantitatively for the stimulated acinar Na secretion²¹.

The Ca-mediated opening of cation channels in the basolateral acinar plasma membrane evoked by CCK or ACh may represent the major control step for acinar fluid secretion in the pancreas and probably also other exocrine glands, such as the salivary glands and the lacrimal glands, where similar types of stimulant-evoked currents have been studied¹⁷, although not at the single-channel level.

This work was supported by an MRC project grant.

Received 19 July; accepted 20 September 1982.

- Mutt, V. & Jorpes, J. E. *Eur. J. Biochem.* **6**, 156–162 (1968).
- Dockray, G. J., Gregory, R. A., Hutchison, J. B., Harris, J. I. & Runswick, M. J. *Nature* **274**, 711–713 (1978).
- Petersen, O. H. *Nature new Biol.* **244**, 73 (1973).
- Nishiyama, A. & Petersen, O. H. *J. Physiol., Lond.* **238**, 145–158 (1974).
- Dodd, J. & Kelly, J. S. *Brain Res.* **205**, 337–350 (1981).
- Petersen, O. H. & Philpott, H. G. *J. Physiol., Lond.* **290**, 305–315 (1979).
- Iwatsuki, N. & Petersen, O. H. *Nature* **268**, 147–149 (1977).
- Petersen, O. H. & Iwatsuki, N. *Ann. N.Y. Acad. Sci.* **307**, 599–617 (1978).
- Maruyama, Y. & Petersen, O. H. *Nature* **299**, 159–161 (1982).
- Peikin, S. R., Costenbader, C. L. & Gardner, J. D. *J. Biol. Chem.* **254**, 5321–5327 (1979).
- Philpott, H. G. & Petersen, O. H. *Pflügers Arch. ges. Physiol.* **382**, 263–267 (1979).
- Jensen, R. T., Lemp, G. F. & Gardner, J. D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2079–2083 (1980).
- Williams, J. A., Korc, M. & Dormer, R. L. *Am. J. Physiol.* **235**, E517–E524 (1978).
- Wakasugi, H. et al. *J. Membrane Biol.* **65**, 205–220 (1982).
- Neher, E. in *Techniques in Cellular Physiology* (ed. Baker, P. F.) Vol. 1, Pt. II, 121, 1–16 (Elsevier, Amsterdam, 1982).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Petersen, O. H. *The Electrophysiology of Gland Cells* (Academic, London, 1980).
- Bolender, R. P. *J. Cell Biol.* **61**, 269–287 (1974).
- Colquhoun, D., Neher, E., Reuter, H. & Stevens, C. F. *Nature* **294**, 752–754 (1981).
- Yellen, G. *Nature* **296**, 357–359 (1982).
- Petersen, O. H. et al. *Phil. Trans. R. Soc. B* **296**, 151–166 (1981).
- Li, J. H.-Y., Palmer, L. G., Edelman, I. S. & Lindemann, B. *J. Membrane Biol.* **64**, 77–89 (1982).
- Palmer, L. G., Li, J. H.-Y., Lindemann, B. & Edelman, I. S. *J. Membrane Biol.* **64**, 91–102 (1982).
- Philpott, H. G. & Petersen, O. H. *Nature* **281**, 684–686 (1979).

The virus of Japanese adult T-cell leukaemia is a member of the human T-cell leukaemia virus group

M. Popovic*, M. S. Reitz Jr*, M. G. Sarngadharan†, M. Robert-Guroff*, V. S. Kalyanaraman†, Y. Nakao‡, I. Miyoshi§, J. Minowada||, M. Yoshida¶, Y. Ito# & R. C. Gallo*

* Laboratory of Tumor Cell Biology, National Cancer Institute, NIH, Bethesda, Maryland 20205, USA

† Department of Cell Biology, Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895, USA

‡ Department of Medicine, Kobe University School of Medicine, Kobe, Japan

§ Department of Internal Medicine, Kochi Medical School, Nankoku Kochi 781-51, Japan

|| Department of Immunology, Roswell Park Memorial Institute, Buffalo, New York 14263, USA

¶ Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan

Department of Microbiology, Kyoto University, Faculty of Medicine, Kyoto, Japan

Human T-cell leukaemia virus (HTLV) has been isolated from several cases of cutaneous T-cell lymphoma in the United States^{1,2}. Subsequently, adult T-cell leukaemia virus (ATLV) was also isolated from several cases of adult T-cell leukaemia in Japan^{3–5}. Since the latter disease has been shown to be correlated $\geq 90\%$ with the presence of high titred natural antibodies^{6–8} to two structural proteins of HTLV [p24 (ref. 9) and p19 (ref. 10)], it was important to compare HTLV and ATL. We report here that by serology of p24 and p19 and by nucleic acid homology, ATL appears to be as closely related to the original isolate of HTLV as different isolates of HTLV (from various regions of the world) are related to each other. ATL is therefore either identical or closely related to HTLV.

HTLV, which has recently been identified outside the United States (refs 8, 11, 12, and M.P. *et al.*, in preparation) can routinely be isolated from HTLV-positive T-cell malignancies now that mature human T cells can be cultured in liquid suspension¹³⁻¹⁵ in the presence of T-cell growth factor^{16,17}. Integrated proviral sequences have been detected in DNA of the HTLV-positive leukaemic T cells but not in DNA from normal uninfected human tissues^{2,8,18,19}, establishing HTLV as an acquired retrovirus of man. Moreover, sera from the HTLV-positive patients contain specific, high-titre antibodies to p24²⁰, the major core protein of HTLV⁹ and to other HTLV structural proteins²¹. Sera from adult T-cell leukaemia (ATL) patients (from south-west Japan, where ATL is endemic)²² also contained high-titre antibodies to HTLV structural proteins⁶⁻⁸. Independently, workers in Japan (including I.M.) described ATL retrovirus particles in a cell line established from a patient with adult T-cell leukaemia (MT-1)³ and in a cord blood T-cell line (MT-2) established by co-cultivation with T cells from a similar patient⁴. Serum antibodies apparently directed against this virus have also been demonstrated, both in Japanese ATL cases and in some normal people in the endemic regions^{3,4}. ATL has since been shown to possess biochemical properties typical of retroviruses⁵ and is present in tumour cell but not normal cell DNA⁵.

Characterization of p24⁹, p19¹⁰, reverse transcriptase²³ and nucleic acids^{2,18} of HTLV shows that it is not closely related to any animal retroviruses other than having some distant similarities of amino acid sequence to p24 of bovine leukaemia virus²⁴. The experiments reported here were designed to determine whether, as suspected, ATL is the same virus as HTLV.

Indirect immunofluorescence (IF) using a monoclonal antibody to HTLV p19¹⁰, competitive radioimmunoassays utilizing homogeneously purified HTLV p24⁹, and nucleic acid assays with HTLV ³H-cDNA and ATL ³²P-cDNA for viral mRNA and proviral DNA^{2,5,18} were used to ascertain the relatedness of ATL to previously described strains of HTLV.

DNA was prepared from the nuclear fraction of MT-1 cells and assayed for the presence of HTLV-related proviral DNA, using highly labelled ³H-cDNA prepared from melittin-permeabilized²⁵ HTLV virus and using DNase-digested calf thymus DNA as a random primer for the endogenous reverse transcriptase. DNA from cells producing HTLV (clone B2) hybridized with HTLV cDNA with a $C_{0t_{1/2}}$ value of ~600 (Fig. 1), commensurate with a copy number of 3-4 per haploid genome and consistent with earlier data^{18,19}. DNA from HL-60, a human myeloid leukaemia cell line²⁶, does not hybridize detectably to HTLV cDNA, also in keeping with earlier results with DNA from many different human and animal cell lines and fresh tissues not producing HTLV¹⁸. In contrast, DNA from MT-1 hybridizes extensively to HTLV cDNA. At the highest C_{0t} value tested (38,000), the per cent hybridization had not reached an apparent plateau (Fig. 1a), but was already 80% of the maximum value observed with clone B2 DNA. Due to the lengthy incubation period required, we did not test higher C_{0t} values. The high $C_{0t_{1/2}}$ of the reaction with MT-1 DNA (~1.7 × 10⁴) is indicative of a copy number sufficiently low (one per eight or nine haploid genomes) to suggest that less than every cell in this line is infected. This is in keeping with earlier data on viral protein expression³. The high degree of homology to HTLV indicates that the detected provirus is

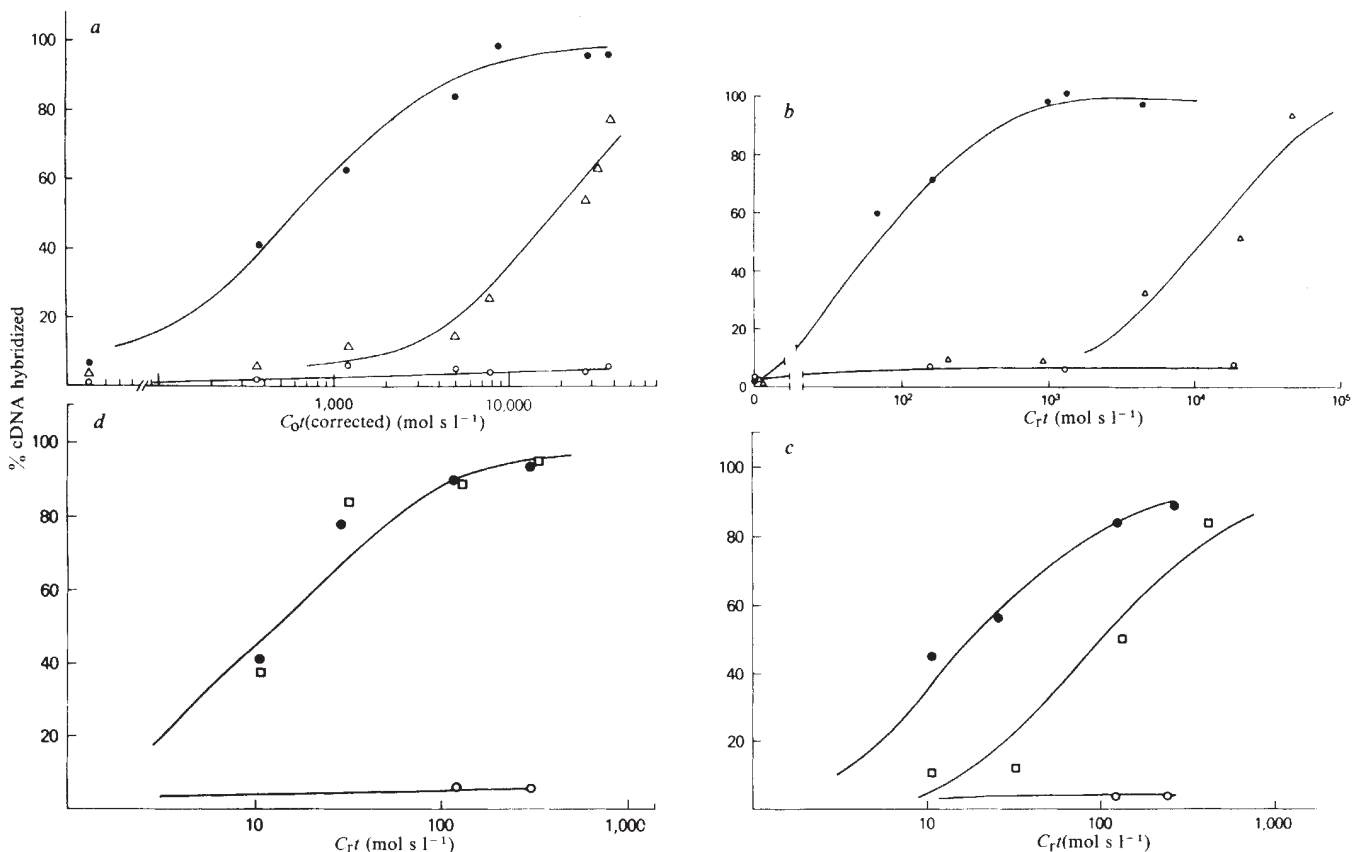
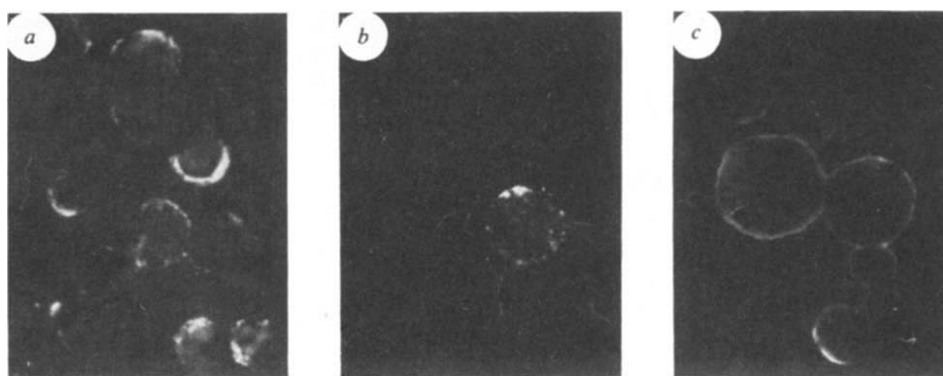


Fig. 1 Comparative analyses of HTLV and ATL by molecular hybridization. DNA or RNA from the indicated cell lines were hybridized with HTLV ³H-cDNA (a-c) or ATL ³²P-cDNA (d) to the indicated C_{0t} (corrected) or C_{rt} and assayed for hybrid formation by digestion with S_1 nuclease as described previously¹⁸. Each point was determined with 500-1,000 c.p.m. cDNA and was corrected for the zero time point (5-8%) and normalized so that the homologous value = 100% (actual value 45-60%). DNA or RNA were from: clone B2 (●), MT-1 (Δ), MT-2 (□) and HL-60 (a) or CR-B cells (○) (b-d). a, ³H-HTLV cDNA was hybridized to the indicated DNA (600 μg per point in 25 μl). b, ³H-HTLV cDNA was hybridized to the indicated RNA. Clone B2 and CR-B cell RNA were poly(A)-selected and present at 25 μg per 80 μl; MT-1 RNA was unselected and present at 57 μg per 25 μl. c, ³H-HTLV cDNA was hybridized to the indicated RNA. MT-2 RNA was not poly(A)-selected and present at 10 μg per 25 μl. d, ³²P-ATLV cDNA was hybridized to the indicated RNA.

Fig. 2 Indirect immunofluorescence assay using monoclonal anti-HTLV p19. Cells were washed and fixed for 10 min with 50% methanol/50% acetone as described previously¹⁰. The indirect immunofluorescence assay using mouse ascites fluid containing monoclonal anti-p19 and fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of sheep anti-mouse IgG has also been described elsewhere¹⁰. *a*, HUT102, clone A9 cells. *b*, MT-1 cells following treatment with 60 $\mu\text{g ml}^{-1}$ IdUrd for 24 h and an additional 24 h in culture in the absence of IdUrd. *c*, MT-2 cells.



identical to HTLV or is a very closely related strain of the HTLV group. Total cytoplasmic RNA was also prepared from MT-1 cells and assayed for the presence of HTLV mRNA. At sufficient $C_{\text{r}}t$ values, similar levels of hybridization are attained with MT-1 RNA and RNA from HTLV-producing cells (Fig. 1*b*), confirming that HTLV is present in MT-1 cells.

In separate experiments, HTLV and ATL V cDNA⁵ were hybridized to RNA from a second cell line producing ATL V, and the values obtained were compared with those obtained with RNA from clone B2. As shown in Fig. 1*c*, HTLV cDNA hybridizes to the same extent to both RNAs, indicating that MT-2, like MT-1, contains sequences extensively related to HTLV. Moreover, ATL V cDNA also hybridizes to the same extent to both RNAs (Fig. 1*d*), confirming the close relatedness of ATL V to HTLV in reciprocal hybridization experiments. The difference in the rates of hybridization of HTLV and ATL V cDNA to MT-2 RNA (Fig. 1*c, d*) is noteworthy. HTLV ³H-cDNA is randomly primed and presumably reasonably representative of the RNA genome, while ATL V ³²P-cDNA⁵ is primarily 'strong stop' cDNA, specific to the 5' end of the RNA (M.Y., unpublished results). The disparity in the rates of hybridization of the two cDNAs suggests that MT-2 cells express RNA transcripts which consist partly of viral leader sequences and partly of cellular sequences. This interpretation is consistent with the relatively complex pattern observed with Northern blots of MT-2 RNA using ATL V cDNA⁵, where several RNA species are evident in addition to the two viral mRNAs observed with MT-1 RNA.

MT-1 cells were assayed for the presence of p19 by indirect immunofluorescence using a highly specific monoclonal antibody¹⁰. Anti-p19 antibody specifically labelled all six cell lines previously shown to contain HTLV (not shown), as determined by virus isolation, detection of HTLV nucleic acids and detection of antibodies to HTLV in serum from the patient. A battery of HTLV-negative cell lines (a total of 127) which included human normal and neoplastic T and B cells and several human and animal cell lines infected with or producing different animal retroviruses, were always negative. HTLV p19 was detected in a small number (~1%) of MT-1 cells. The number of fluorescent cells increased to ~8% on treatment with iododeoxyuridine (IdUrd). These numbers, although relatively low, are highly reproducible and are consistent with both the low estimated proviral copy number mentioned above and with the results of Hinuma *et al.*³. Virtually all the cells in the T-2 culture express HTLV p19. The range of p19-positive cells by this assay in other HTLV-producing cell lines is 5–95%. The highest percentage of anti-p19-labelled cells (besides that with MT-2) was observed with clones B-2 and A-9, derived from the HUT102 cell line (M. Maeda, M.P. and R.G., unpublished). Two additional HTLV-producing cell lines, CTCL-3¹ and CTC-16²⁷, established independently from the same patient (C.R.) as the HUT102 cell line, contained 20 and 38% positive cells, respectively. The proportions of fluorescent cells in CTCL-2² and a newly established HTLV-positive neoplastic human T-cell line, MJ (M.P. *et al.*, in preparation), were 5 and 85%, respectively.

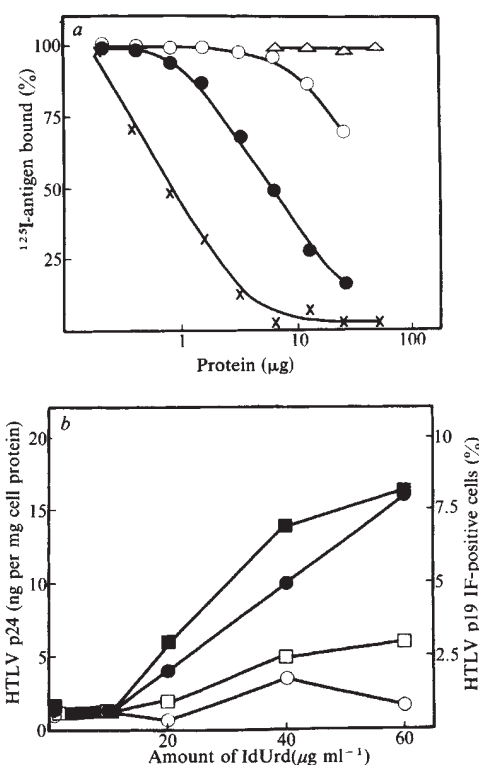


Fig. 3 Competition radioimmunoassay of HTLV p24. *a*, Homologous competition radioimmunoassay of HTLV p24. Competition radioimmunoassays were carried out with ¹²⁵I-labelled HTLV p24 and a limiting dilution of a rabbit antibody to HTLV. Serial dilutions (100 μl) of the solubilized cells in buffer 1 (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.3% Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride and 2 mg ml⁻¹ bovine serum albumin) were incubated with rabbit antibody to HTLV (50 μl containing 1:50 diluted normal rabbit serum) for 1 h at 37 °C. Labelled HTLV p24 (8,000 c.p.m.) in 50 μl of buffer 1 was then added and the mixture was further incubated at 37 °C for 2 h and overnight at 4 °C. A 20-fold excess of goat anti-rabbit IgG was then added and the volume was made up to 1 ml in buffer 1. The samples were further incubated at 37 °C for 1 h, 2 h at 4 °C and then centrifuged at 2,500 r.p.m. for 5 min in a Beckman centrifuge. The supernatants were aspirated and the radioactivity in the pellet was determined in an LKB ultra-gamma counter. x-x, Clone B2 cells; ●-●, MT-1 24 h after IdUrd induction; ○-○, uninduced MT-1 cells; Δ-Δ, cultured normal human T cells. *b*, IdUrd induction of HTLV p19 and p24 in MT-1 cells. Expression of HTLV p19 and p24 was quantified according to methods described in Figs 2 and 3 legends. Cultured MT-1 cells were exposed to different concentrations of IdUrd for 24 h and the cells were washed free of IdUrd and HTLV p19 and p24 were measured 0 and 48 h later. ○, ●, p24; □, ■, p19. Open symbols, 0 time after the 24 h IdUrd treatment; filled symbols, 48 h after removal of the IdUrd from the culture medium.

These cell lines, like CTCL-3 and CTC-16, were established from the peripheral blood of patients with leukaemia or lymphoma involving relatively mature T cells. An example of the immunofluorescence obtained using monoclonal anti-p19 antibody on MT-1 and MT-2 cells and a positive control cell line, clone A9, is shown in Fig. 2.

A specific competition radioimmunoassay has been described for the HTLV core protein, p24⁹, which can detect as little as 0.05 ng of p24. This precipitation assay uses ¹²⁵I-labelled homogeneous HTLV p24 and a hyperimmune rabbit antiserum against detergent-disrupted HTLV. Competition is observed only with proteins from disrupted HTLV or HTLV-producing cells. As shown in Fig. 3a, cell extracts from both MT-1 and the HTLV-producing cell line, clone B-2, competed in the HTLV p24 radioimmunoprecipitation assays. The competition with IdUrd-induced MT-1 cells was about 10-fold higher than with untreated MT-1 cells, and the slope and end point of the competition curves with clone B2 and induced MT-1 were the same. The p24 determinants of HTLV and ATL which are recognized by this antiserum are thus indistinguishable. No competition was observed with extracts from HTLV-negative control cells, which included cultured human normal mature T cells and leukaemic T-cell lines previously shown to be HTLV negative.

The kinetics of induction of expression of p19 and p24 were analysed in parallel after treatment of MT-1 cells with IdUrd for 24 h. As shown in Fig. 3b, the induction of both proteins was time-dependent and increased with increasing concentrations of inducer. There was a slight increase in the expression of both p19 and p24 immediately following the 24-h treatment of MT-1 cells with IdUrd. However, a substantial increase in the expression of both proteins was observed 48 h after IdUrd treatment. At the highest IdUrd concentration tested (60 µg ml⁻¹), the p19 expression increased about 8-fold while a 16-fold increase was detected for p24 compared with the untreated control cells. The expression of both these proteins therefore increased simultaneously on IdUrd treatment of MT-1 cells. It is therefore likely that the 'adult T-cell leukaemia-associated antigen' or 'ATLA' in MT cells detected following induction with IdUrd and using natural antibodies in sera of Japanese ATL patients³, represents one of these previously described HTLV proteins^{9,10}.

We have previously shown that >90% of tested Japanese patients with ATL also contained antibodies to HTLV p24⁶⁻⁸. A subsequent more extensive screen of a large number of sera of Japanese ATL patients has substantiated the earlier finding²⁸. In addition, 25% of close relatives of ATL patients and about 10% of individuals in the area endemic for ATL also have antibodies to HTLV p24. In contrast, none of 40 normal random sera from the non-endemic regions of Japan had anti-HTLV p24 antibodies.

We conclude from the data presented here that the antigens reacting with the antibodies in the sera of most Japanese ATL patients are highly cross-reactive with HTLV proteins p19 and p24. Two cell lines from such patients (MT-1 and MT-2), which contain ATL RNA and proviral DNA⁵, also contain viral nucleotide sequences which are virtually indistinguishable from those of HTLV. MT-1 (which produces ATL³) expresses two antigenic determinants indistinguishable from those of HTLV p24 and p19. Virtually all MT-2 cells (which produce ATL⁴) express HTLV p19. Moreover, three other Japanese ATL cell lines established in our laboratory (M.P. *et al.*, in preparation) and two other similar lines established in Japan (S. Morikawa, personal communication) also express antigenic determinants indistinguishable from those of HTLV p24 and p19. The widespread occurrence of antibodies in the Japanese ATL sera strongly reactive with HTLV p24 further strengthens our conclusion that the retrovirus associated with Japanese ATL is identical or very closely related to previously described strains of HTLV^{1,2,18} which have been isolated and characterized from patients in the US with leukaemias and lymphomas involving mature T cells, and establish ATL within the HTLV group.

We thank M. Jarvis, E. Richardson and E. Shepard for technical assistance. Fixed cells from MT-2 were provided by Dr Y. Hinuma. M.P. was a recipient of the American Cancer Society, Eleanor Roosevelt International Cancer Fellowship awarded by the International Union Against Cancer.

Received 23 July; accepted 26 August 1982.

- Poiesz, B. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7415-7419 (1980).
- Poiesz, B. J., Ruscetti, F. W., Reitz, M. S., Kalyanaraman, V. S. & Gallo, R. C. *Nature* **294**, 268-271 (1981).
- Hinuma, Y. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 6476-6480 (1981).
- Miyoshi, I. *et al. Nature* **294**, 770-771 (1981).
- Yoshida, M., Miyoshi, I. & Hinuma, Y. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2031-2035 (1982).
- Robert-Guroff, M. *et al. Science* **215**, 975-978 (1982).
- Kalyanaraman, V. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1653-1657 (1982).
- Gallo, R. C. *et al. in Biochemical and Biological Markers on Neoplastic Transformation* (Plenum, New York, in the press).
- Kalyanaraman, V. S., Sarngadharan, M. G., Poiesz, B., Ruscetti, F. W. & Gallo, R. C. *J. Virol.* **38**, 906-915 (1981).
- Robert-Guroff, M., Ruscetti, F. W., Posner, L. E., Poiesz, B. J. & Gallo, R. C. *J. exp. Med.* **154**, 1957-1964 (1981).
- Catovsky, D. *et al. Lancet* **i**, 639-643 (1982).
- Blattner, W. A. *et al. Int. J. Cancer* (in the press).
- Morgan, D. A., Ruscetti, F. W. & Gallo, R. C. *Science* **193**, 1007-1008 (1976).
- Ruscetti, F. W., Morgan, D. A. & Gallo, R. C. *J. Immun.* **119**, 131-138 (1977).
- Poiesz, B. J., Ruscetti, F. W., Mier, J. W., Woods, A. M. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6815-6819 (1980).
- Mier, J. W. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6134-6138 (1980).
- Mier, J. W. & Gallo, R. C. *J. Immun.* **128**, 1122-1127 (1982).
- Reitz, M. S., Poiesz, B. J., Ruscetti, F. W. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1887-1891 (1981).
- Gallo, R. C. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Kalyanaraman, V. S., Sarngadharan, M. G., Bunn, P. A., Minna, J. D. & Gallo, R. C. *Nature* **294**, 271-273 (1981).
- Posner, L. E. *et al. J. exp. Med.* **154**, 333-346 (1981).
- Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K. & Uchino, H. *Blood* **50**, 481-492 (1977).
- Rho, H. M., Poiesz, B., Ruscetti, F. W. & Gallo, R. C. *Virology* **112**, 355-360 (1981).
- Oroszlan, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1291-1294 (1982).
- Boone, L. R. & Skalka, A. *Proc. natn. Acad. Sci. U.S.A.* **77**, 847-851 (1980).
- Collins, S., Gallo, R. C. & Gallagher, R. E. *Nature* **270**, 347-349 (1977).
- Uchiyama, T., Broder, S., Bonnard, G. & Waldmann, T. *Trans. Am. Ass. Phys.* **93**, 251-262 (1980).
- Robert-Guroff, M. *et al. J. exp. Med.* (in the press).

Human T-cell clones recognize chemically synthesized peptides of influenza haemagglutinin

Jonathan R. Lamb*, David D. Eckels*, Phil Lake*, James N. Woody† & Nicola Green‡§

* Division of Immunologic Oncology, Vincent T. Lombardi Cancer Research Center, Department of Pediatrics and Microbiology, Georgetown University School of Medicine, Washington, DC 20007, USA

† Naval Medical Research Institute, Bethesda, Maryland 20814, USA

‡ Committee for the Study of Molecular Genetics, Research Institute of Scripps Clinic, La Jolla, California 92037, USA

The continual variation in the antigenic structure of the haemagglutinin (HA) molecule is associated with the appearance of new epidemics or pandemics of influenza in immune populations¹. The detailed analysis of HA structure has provided the molecular basis of this phenomenon²⁻⁴ and while four antibody-binding sites have been suggested⁴, the epitopes of HA that are recognized by T cells remain undefined. Using chemically synthesized peptides corresponding to sequences of the HA molecule⁵, we have investigated the antigenic determinants recognized by long-term T-lymphocyte lines and clones induced with HA and maintained in T-cell growth factor (TCGF or interleukin-2, IL-2). The data suggest that HA-specific T-cell populations contain the repertoire to respond to all the synthetic peptides analysed. However, when responses are analysed at the clonal level, one peptide located at the carboxy-terminus of the HA1 molecule and discrete from the proposed antibody-binding sites⁴ seems to be immunodominant.

§ To whom correspondence should be addressed.

Peripheral blood lymphocytes (PBL) from adult volunteers selected for known responses to strain A influenza virus (A/Texas/1/77; H₃N₂) were cultured with HA isolated from this subtype. After 6 days, cultures were either cloned by limiting dilution in the presence of TCGF, irradiated autologous PBL and specific antigen as previously described⁶, or maintained as cell lines. The T lymphocyte clones and lines were maintained in continuous culture for 8 weeks and then assayed for responsiveness to specific antigen in the presence of antigen-presenting cells as determined by tritiated thymidine (³H-TdR) incorporation.

Normal PBL responded to the intact virus and to the HA molecule of A/Texas/1/77, as did long-term influenza and HA lines induced with the same viral subtype (Table 1). The antigenic specificity of these lines was confirmed by their failure to respond to the unrelated strain B influenza virus, B/Singapore/222/79. Both normal PBL and T-cell lines in the presence of antigen-presenting cells responded to each of the synthetic peptides (Fig. 1) tested over a dose range of 0.01–10 µg ml⁻¹. Although these peptides were synthesized according to the sequences of influenza strain X-47 (ref. 7), in most cases they were identical to corresponding regions of A/Texas/1/77 (ref. 8). Both viruses are H3N2, and comparison of their sequences reveals only a few amino acid differences. It is evident that peptide 20, which corresponds to the carboxy-terminus of HA1, induced the maximum proliferative response (Table 1). Interestingly, this peptide is distant from the four proposed antibody-binding sites. Although these sites are able

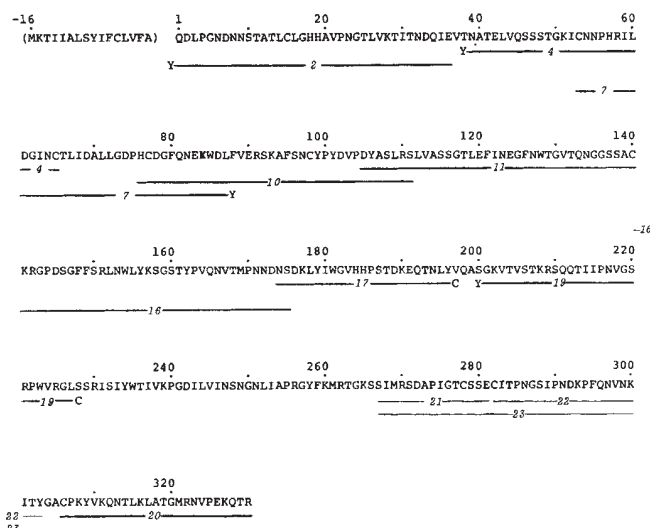


Fig. 1 Amino acid sequence of the HA1 molecule of X-47 as predicted from the nucleotide sequence⁷. The regions of the molecule synthesized are underlined and numbered. C and Y at the end of the peptide indicate, respectively, the addition of a cysteine or tyrosine not present in the primary sequence.

Table 1 Reaction of normal peripheral blood lymphocytes and influenza A virus- and HA-specific long-term T-cell lines with synthetic peptides of the HA1 molecule

Synthetic peptides of HA-1 molecule	Normal PBL	Lymphocytes Influenza A line	HA line
2	2,388 (206)	10,308 (1,737)	1,797 (326)
4	3,461 (171)	1,008 (387)	2,216 (321)
7	6,204 (494)	7,885 (515)	1,497 (296)
10	3,924 (583)	7,287 (1,005)	2,088 (64)
11	5,960 (926)	11,359 (2,179)	1,349 (321)
16	2,359 (639)	9,173 (780)	1,439 (366)
17	1,667 (186)	6,683 (566)	1,470 (264)
19	2,267 (334)	10,091 (25)	1,131 (381)
20	9,061 (1,737)	30,266 (6,240)	3,549 (272)
21	2,460 (373)	5,871 (764)	1,622 (196)
22	4,733 (445)	2,065 (245)	1,378 (174)
23	3,017 (308)	8,743 (952)	2,094 (140)
HA molecule (A/Texas/1/77; H ₃)	6,514 (512)	16,792 (1,808)	2,326 (452)
Intact virus (A/Texas/1/77)	16,017 (2,816)	40,645 (9,307)	9,173 (1,072)
Influenza B strain (B/Singapore/222/79)	22,085 (4,969)	57 (10)	27 (16)
Medium alone	21 (5)	20 (6)	21 (6)
TCGF	—	3,481 (365)	1,829 (52)

Normal human peripheral blood lymphocytes (PBL, 2.5×10^5 ml⁻¹) were cultured with either the synthetic peptides of the HA1 molecule, the purified HA molecule (gift of Dr R. G. Webster), or intact influenza A (A/Texas/1/77) or B (B/Singapore/222/79) strain virus for 6 days in RPMI-1640 (Gibco) supplemented with 10% pooled A⁺ serum. The concentration of antigen used for the synthetic peptides and HA was 0.01–10 µg ml⁻¹, and 0.05–50 haemagglutinating units (HAU) ml⁻¹ for the intact viruses. To induce T-cell lines, PBL were cultured with intact A/Texas/1/77 or the purified HA molecule of A/Texas, and after 6 days the lymphoblasts were enriched on a discontinuous Percoll gradient. These blast cells (10^5 ml⁻¹) were expanded as long-term T-cell lines with filler cells (irradiated (2,500 rad) autologous PBL at 5×10^5 ml⁻¹) and specific antigen in the presence of T-cell growth factor. TCGF was prepared by culturing PBL (1×10^6 ml⁻¹) with 0.1% purified phytohaemagglutinin-P (PHA-P; Difco) in RPMI-1640 supplemented with 1% autologous plasma¹⁷. After 48 h, supernatants were collected and assayed for their ability to support growth of a TCGF-dependent line as described previously⁶. The lines were maintained with filler cells and antigen every 7 days and TCGF every 3 days. Influenza A virus and HA-specific T-cell lines (2.5×10^4 ml⁻¹) were cultured with the panel of antigens in the presence of irradiated autologous PBL (1.25×10^5 ml⁻¹) for 72 h, pulsed for 8–16 h with 1.0 µCi of tritiated methylthymidine (³H-TdR; NEN) and collected on to glass fibre filters. Proliferation, as correlated with ³H-TdR incorporation, was measured by liquid scintillation spectroscopy⁶. Results are expressed as mean c.p.m. (±s.e.m.) of triplicate cultures. Only the highest proliferative response in the antigen dose-response curve is shown. As controls, the responses to medium alone, TCGF and B/Singapore were determined.

to induce proliferation, it appears that they are not the immunodominant region of the HA molecule for T-cell responses. In comparison, studies on immune responses to myoglobin in mice have demonstrated that the five antigenic sites recognized by antibody are also the primary inducers of T-cell proliferation in cell populations^{9,10} and long-term cultures¹¹. Furthermore, it was reported that peptides representing B-cell non-antigenic parts of the molecule were unable to induce proliferation¹⁰, which may reflect the fact that regions of myoglobin are phylogenetically conserved and are thus immunologically silent. However, our findings demonstrate that the entire HA molecule as defined by the synthetic peptides used in these studies can induce T-cell proliferation with none of the peptides behaving as nonimmunogens, as was observed in myoglobin¹⁰, or actively inducing suppression, as has been reported for lysozyme^{12,13}. Analysis of the activity of smaller peptides of HA, however, may allow such regions to be identified.

The fine specificity of antigen recognition by HA-specific T cells was investigated at the clonal level using chemically synthesized peptides of HA1 (Table 2). One of the four T-lymphocyte clones (HA1.9) proliferated in response to the HA molecule and the intact influenza A virus, but failed to respond to B/Singapore or to the panel of synthetic peptides. Presumably, this clone recognizes determinants that reside in the sequences not covered by overlapping peptides (Fig. 1). The remaining three clones analysed (HA1.4, HA1.7 and HA2.43) responded only to peptide 20 and not to the predominant antibody-binding sites. Comparable to this observation is a report on the clonal analysis of T-cell recognition of the antigenic determinants of myoglobin, where two clones specific for the fragment 1–55 failed to respond to peptide 15–22, which contains the only antibody-binding site¹¹.

To define the epitope specificity of T-cell antigen recognition, clones HA1.4, HA1.7 and HA2.43 were cultured with the intact viruses A/Japan/305/57 (H2N2) and A/Texas/1/77 (H3N2). Neither clone HA1.4 nor HA1.7 responded to A/Japan, but both proliferated in response to stimulation with A/Texas (Table 3), suggesting that these two clones recognize a variant region of the carboxy-terminus of HA1. Any one of the amino acid substitutions between A/Japan and A/Texas occurring within the sequence of peptide 20 at residues 312–314, 316, 321 and 326–329 (Fig. 2) would be able to confer the specificity of these clones for A/Texas as opposed to A/Japan, as it has been reported that T-cell activation may

Table 2 Recognition of synthetic peptides by HA-specific human T-lymphocyte clones

Synthetic peptides of HA1 molecule	HA1.4	HA1.7	Clone no.	HA1.9	HA2.43
2	61 (5)–92 (16)	17 (6)–59 (13)		25 (11)–57 (15)	17 (5)–57 (14)
4	24 (2)–54 (14)	17 (1)–31 (11)		25 (5)–37 (9)	49 (9)–81 (9)
7	33 (7)–136 (50)	13 (4)–38 (1)		16 (8)–51 (19)	10 (1)–61 (7)
10	31 (9)–96 (72)	27 (3)–38 (5)		26 (9)–52 (14)	28 (4)–74 (9)
11	15 (3)–47 (5)	19 (5)–81 (25)		14 (3)–37 (1)	17 (4)–37 (9)
16	16 (4)–65 (45)	15 (4)–26 (10)		14 (8)–93 (51)	15 (4)–43 (8)
17	24 (5)–40 (8)	20 (5)–45 (23)		20 (5)–45 (23)	18 (7)–19 (2)
19	27 (5)–51 (4)	27 (5)–67 (14)		14 (2)–49 (12)	31 (5)–61 (14)
20	69 (46)–3,591 (22)	25 (4)–2,846 (158)		33 (15)–83 (23)	886 (30)–13,662 (1,514)
21	21 (2)–48 (2)	22 (2)–44 (15)		16 (4)–43 (7)	17 (2)–31 (6)
22	18 (4)–44 (7)	19 (4)–39 (7)		16 (2)–28 (4)	11 (3)–21 (3)
23	12 (1)–22 (3)	48 (9)–63 (6)		21 (3)–52 (16)	9 (2)–39 (3)
HA molecule (A/Texas/1/77; H ₃)	2,607 (282)	3,073 (180)		10,440 (566)	12,038 (280)
Intact virus (A/Texas/1/77)	7,772 (732)	3,009 (99)		22,889 (6,751)	14,401 (926)
Influenza B strain (B/Singapore/222/79)	37 (11)	29 (2)		43 (6)	18 (2)
Medium alone	20 (1)	27 (4)		27 (4)	29 (2)
TCGF	3,813 (366)	1,018 (29)		8,099 (521)	1,276 (25)

HA-specific human T-lymphocyte clones were isolated as described previously for influenza A virus⁶. Briefly PBL were cultured for 6 days with 0.1 $\mu\text{g ml}^{-1}$ HA. The lymphoblasts enriched on a discontinuous Percoll gradient and resuspended at 333 cells ml^{-1} in RPMI-1640 supplemented with 10% A⁺ serum and 20% TCGF, were plated out at one cell every third well in Terasaki trays with 10⁴ irradiated autologous PBL and 0.1 $\mu\text{g ml}^{-1}$ HA. After 7 days, growing clones were transferred to 96-well microtitre trays and then to 24-well trays. Then they were expanded in 25-cm² tissue culture flasks. At each transfer the T-lymphocyte clones received fresh TCGF and irradiated autologous PBL, together with specific antigen. The clones were maintained with 20% TCGF every 3–4 days and irradiated autologous PBL and HA were added every 7 days. Before use in proliferation assays, the clones were allowed to rest for 6–7 days after the addition of filler cells. HA-specific clones ($2.5 \times 10^4 \text{ ml}^{-1}$) were cultured with the synthetic peptides of HA1 molecule (0.01–10 $\mu\text{g ml}^{-1}$), HA molecule (0.01–10 $\mu\text{g ml}^{-1}$), A/Texas/1/77 (0.05–50 HAU ml^{-1}) or B/Singapore/222/79 (0.05–50 HAU ml^{-1}) in the presence of irradiated autologous sheep erythrocyte rosette-negative (E[−]) cells ($2.5 \times 10^4 \text{ ml}^{-1}$) for 72 h, and proliferation measured by ³H-TdR incorporation. E[−] cells were fractionated as described previously⁶. Results are expressed as mean c.p.m. ($\pm$ s.e.m.) of triplicate cultures. Both the lowest and highest proliferative responses for the antigen concentrations used are shown for the synthetic peptides.

Table 3 Specificity of T-cell clones for A/Japan/305/57 (H2N2) and A/Texas/1/77 (H3N2) subtypes of influenza A virus

Antigen	HA1.4	Clone no. HA1.7	HA2.43
Peptide 20	1,145 (138)–15,265 (1,356)	1,047 (207)–17,408 (1,799)	620 (65)–3,373 (481)
A/Texas/1/77	645 (134)–15,561 (438)	975 (251)–17,406 (822)	472 (109)–3,089 (396)
A/Japan/305/57	55 (29)–91 (21)	63 (49)–123 (16)	274 (28)–2,620 (229)
B/Singapore/222/79	19 (5)–42 (6)	25 (3)–56 (2)	27 (3)–49 (9)
Medium alone	21 (3)	55 (23)	11 (3)
TCGF	1,409 (24)	1,982 (57)	1,472 (139)

HA-specific clones ($2.5 \times 10^4 \text{ ml}^{-1}$) were cultured with peptide 20 (0.01–10 $\mu\text{g ml}^{-1}$), A/Texas/1/77, A/Japan/305/57 or B/Singapore/222/79 in the presence of irradiated autologous E[−] cells ($2.5 \times 10^4 \text{ ml}^{-1}$) for 72 h. The intact influenza viruses were added at concentrations of 0.05–50 HAU ml^{-1} . Proliferation was determined by the incorporation of ³H-TdR. Results are expressed as described in Table 2 legend.

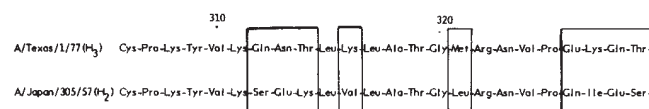


Fig. 2 Amino acid substitutions between A/Japan/305/57 and A/Texas/1/77 occurring within the sequence of peptide 20. The sequence comparison was derived from Gething *et al.*². The sequence numbering is according to Min Jou *et al.*⁷ in order to conform with our peptide designations. The boxed areas indicate substitutions.

be sensitive to a single amino acid substitution^{14,15}. In contrast to clones HA1.4 and HA1.7, clone HA2.43 recognized both viral subtypes, which implies that it recognizes an invariant sequence of peptide 20, or less likely that the amino acid substitutions between A/Japan and A/Texas are insufficient to cause modification of the molecule to influence T-cell antigen recognition¹⁴.

The findings reported here do not resolve the issue of the nature of T-cell recognition in terms of a defined amino acid sequence or its dependency on tertiary structure¹⁶. In an attempt to define the epitope sequence, subunits of peptide 20 were generated with the four carboxy-terminal amino acids and increasing the subunit sequence progressively by four amino acids until the complete peptide was synthesized. Unlike the intact peptide, however, none of the subunits induced proliferation of the clones (data not shown). This result does not

distinguish whether the amino terminus of peptide 20 is the precise sequence recognized or whether it dictates the conformation of the native peptide necessary for T-cell recognition. An alternative explanation is that the subunits themselves cannot adopt the same conformation as in the intact peptide, therefore they are no longer recognized by the T cells.

The technical assistance of Ms B. Ketteter is acknowledged. This work was supported by the Office of Naval Research Contracts N000-14-77C0748, 77C0747 and 80K0909 and the Naval Medical Research and Development Command Work Unit MOO95PN001. We thank Dr R. A. Lerner for helpful discussions in the preparation of this manuscript, which is publication no. 2783 from the Research Institute of Scripps Clinic.

Received 2 August; accepted 2 September 1982.

1. Webster, R. G. & Laver, W. G. in *The Influenza Viruses and Influenza* (ed. Kilbourne, E. D.) 269–314 (Academic, New York, 1975).
2. Gething, J. J., Bye, J., Skehel, J. & Waterfield, M. *Nature* **287**, 301–306 (1980).
3. Wilson, I. A., Skehel, J. J. & Wiley, D. C. *Nature* **289**, 366–373 (1981).
4. Wiley, D. C., Wilson, I. A. & Skehel, J. J. *Nature* **289**, 373–378 (1981).
5. Green, N. *et al. Cell* **28**, 477–487 (1982).
6. Lamb, J. R. *et al. J. Immun.* **128**, 233–238 (1982).
7. Min Jou, W. *et al. Cell* **19**, 683–693 (1980).
8. Laver, W. G., Air, G. M., Doppeide, T. A. & Ward, C. W. *Nature* **283**, 454–457 (1980).
9. Berzofsky, J. A. in *Biological Regulation and Development* Vol. 11 (ed. Goldberger, R. F.) 467–594 (Plenum, New York, 1980).
10. Okuda, K., Twining, S. S., David, C. S. & Atassi, M. Z. *J. Immun.* **123**, 182–188 (1979).
11. Infanti, A. J., Atassi, M. Z. & Fathman, C. G. *J. exp. Med.* **154**, 1342–1356 (1981).
12. Adorini, L., Harvey, M. A., Miller, A. & Sercarz, E. E. *J. exp. Med.* **150**, 293–307 (1979).

13. Yowell, R. L., Areano, B. A., Miller, A. & Sercarz, E. E. *Nature* **279**, 70–72 (1979).
 14. Thomas, D. W. *et al. J. exp. Med.* **152**, 620–632 (1980).
 15. Matis, L. A. *et al. J. Immun.* **128**, 2439–2446 (1982).
 16. Buchmüller, Y. & Corradin, G. *Eur. J. Immun.* **12**, 412–416 (1982).
 17. Inouye, H. *et al. Scand. J. Immun.* **12**, 149–154 (1980).

Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy

J. M. Murray*†, K. E. Davies*, P. S. Harper†, L. Meredith†, C. R. Mueller‡ & R. Williamson*

* Department of Biochemistry, St Mary's Hospital Medical School, University of London, London W2 1PG, UK

† Department of Medical Genetics, University Hospital of Wales, Cardiff CF4 4XN, UK

‡ Department of Human Genetics, University of Freiburg, 7800 Freiburg, FRG

Duchenne muscular dystrophy (DMD) is one of the most common and serious human X-linked disorders. It occurs at a frequency of up to 1 in 5,000 newborn males in most populations studied, with about one-third of all cases due to new mutations¹. The primary biochemical defect remains unknown, and no proven prenatal diagnostic test exists, although raised serum creatine kinase levels act as a somewhat equivocal guide to carrier females². Previous studies have shown no measurable genetic linkage of the DMD locus with any X-chromosome marker^{3,4}. Therefore, if a cloned sequence of the X chromosome could be used to define the locus, and to provide a closely linked set of markers, it would be of considerable importance in the prediction and prevention of DMD, as well as a step towards identifying the basic biochemical defect causing the disease. We present here evidence of an X-chromosome sequence, defined by its restriction enzyme polymorphism, that is loosely linked to DMD, at a distance of approximately 10 centimorgans, as determined by studies on nine informative families. The polymorphism occurs in 29% of women in a control London population and in 22% of carriers for DMD. The linkage data support cytogenetic evidence that DMD is on the short arm of the X chromosome. The object of this letter is to encourage others to make use of our probe, which seems to be linked to the DMD locus.

Restriction fragment length variation results from changes in the primary DNA sequence, such as single base changes introducing or deleting a restriction site, or sequence deletions, additions or translocations affecting the length of DNA between sites. These DNA sequence polymorphisms are inherited in a mendelian fashion and may be used in conventional linkage studies^{5–8}. Variations in restriction sites around the human β -globin genes have been estimated to occur once in every 100 base pairs⁹. Polymorphisms are sufficiently frequent in the human genome to assume that one will be found adjacent to any random cloned DNA probe several kilobases (kb) in length. A set of such sequences can be used to construct a linkage map of the entire human genome, or of a particular chromosomal region^{6,10–13}.

We have previously cloned a collection of sequences from the human X chromosome in λ phage after sorting using a cytofluorimeter; approximately 50,000 clones were obtained, sufficient to ensure that >90% of sequences from the X chromosome are represented in the library¹⁴.

Single-copy sequences were identified in the X-chromosome library using Benton–Davis screening¹⁵ to total labelled human DNA; clones containing repetitive elements present at >100 copies in the genome hybridize and are labelled in the reannealing conditions used¹⁶. One of the single-copy clones obtained, given the laboratory acronym λ RC8, was characterized by

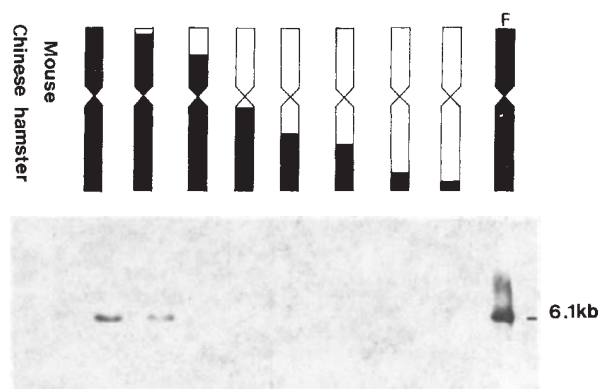


Fig. 1 Localization of λ RC8 to the region Xp21–Xp223 using *Eco*RI-digested DNA from a panel of rodent–human cell hybrids. Positioned above each lane is a representation of the human X chromosome, shaded to show the portion which is present in the hybrid cell line from which the DNA was obtained (from left to right): Chinese hamster parental cell line, mouse parental cell line; hybrids containing part or all of the human X chromosome: Xpter–Xqter, Xp223–Xqter, Xp21–Xqter, Xq12–Xqter, Xq21–Xqter, Xq22–Xqter, Xq26–Xqter, Xq28–Xqter, fibroblast cell line (F). These cell lines were characterized cytogenetically and by isozyme markers in the department of Genetics, University of Freiburg. High molecular weight total DNA was extracted, digested and analysed by Southern blotting as previously described^{23,24}. Blots were washed in 1×SSC at 65°C and autoradiographed at –70°C for 72 h.

hybridization to *Eco*RI-digested human DNA, DNA from various rodent–human hybrid cell lines containing X chromosomes or fragments as the only human genome component, and mouse DNA using Southern blots¹⁷.

This clone contains a 6.1-kb insert of human DNA and hybridizes to a single 6.1-kb band in *Eco*RI-digested human genomic DNA, and DNA from HORL9.X cells (a mouse–human hybrid cell line in which the only human chromosome is the X chromosome), but not to mouse DNA (Fig. 1). It hybridizes to 46XY, 46XX and 48XXXX genomic DNAs with an intensity relative to the number of X chromosomes present (not shown), demonstrating that it is X-chromosome specific. In hybridizations to total human DNA it gives a slight background after moderate stringency washes (1×SSC) which disappears after washing filters at a higher stringency (0.1×SSC) and does not interfere with the analysis reported here.

Somatic cell hybrids were used to locate the DNA sequence cloned in λ RC8 to a region of the X chromosome. Hybridization of the clone to DNA samples from various rodent–human cell lines containing portions of the human X chromosome localized λ RC8 to the region Xp21–Xp223 (Fig. 1). There was no correlation between hybridization and the spectrum of autosomes present in these cell lines.

Table 1 Linkage data for Duchenne muscular dystrophy, probe λ RC8, Xg blood group and colour blindness (CB)

	DMD– λ RC8 <i>n</i> = 9	DMD–Xg <i>n</i> = 10	DMD–CB <i>n</i> = 3	λ RC8–Xg <i>n</i> = 4
θ				
0.01	+0.393	–13.403	–9.781	–0.711
0.05	+1.535	–6.667	–5.018	–0.099
0.10	+1.766	–3.986	–3.082	+0.090
0.15	+1.715	–2.573	–2.028	+0.050
0.20	+1.543	–1.683	–1.343	+0.161
0.25	+1.305	–1.085	–0.865	+0.144
0.30	+1.033	–0.675	–0.524	+0.115
0.35	+0.753	–0.394	–0.283	+0.078
0.40	+0.481	–0.203	–0.123	+0.046
0.45	+0.229	–0.078	–0.030	+0.017

Results are expressed as lod scores (log of the odds) for different values of recombination fraction (θ). The number of kindreds informative for each comparison is indicated (*n*).

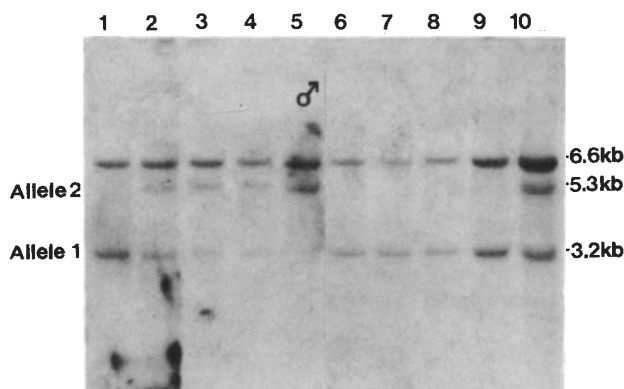


Fig. 2 Variants identified by λ RC8 in *TaqI*-digested total DNA from obligatory carriers of DMD. Sample 5 is from the affected son of obligatory carrier 2. Heterozygotes have bands at 5.3 and 3.2 kb; homozygotes have either the 5.3- or 3.2-kb band. Blood samples (10 ml) were taken from Duchenne patients, carriers and normal controls and total DNA prepared²⁴. *TaqI*-digested DNA (10 μ g) was analysed by Southern blotting^{17,23}. After washing to moderate stringency ($1\times$ SSC), filters were autoradiographed using Fuji X-ray film and intensifying screen for 72 h at -70°C .

λ RC8 was used to identify polymorphisms by screening panels of normal female DNAs digested with different restriction enzymes. It was found to give a variable pattern of bands with *TaqI*-digested total human DNA, hybridizing to the following fragments: an invariant band of 6.6 kb and a second fragment of either 5.3 or 3.2 kb (Fig. 2). The cloned sequence did not identify polymorphisms with 15 human DNAs digested with *MspI*, *XbaI*, *BamHI* or *SstI*.

Females showing hybridization to both 5.3- and 3.2-kb bands are heterozygous for the restriction fragment length polymorphism: they have one X chromosome which gives a 6.6-kb plus a 3.2-kb fragment (pattern 1), and a homologous X chromosome where the *TaqI* site is altered and the probe hybridizes to give fragments of 6.6 kb plus 5.3 kb (pattern 2). In *TaqI* digests of male DNA, λ RC8 only hybridizes to two bands, the 6.6-kb fragment and either the 3.2-kb or the 5.3-kb fragment, never to three fragments. The nature of the polymorphic change has not been investigated but is irrelevant to the linkage analysis. It is only necessary that the alteration is stable and is inherited in a mendelian fashion.

Of a total of 26 *TaqI*-digested normal female and 23 normal male DNAs screened, 7 females were heterozygous (2/1) and 3 males had the rarer allele (2). All the other samples had the common allele (1). Calculated from the males, this gives a gene frequency for the rarer allele of 0.13 and is consistent with the *TaqI* polymorphism identified by λ RC8 behaving as a single-copy X-chromosome sequence.

Families in which DMD occurred in at least two generations, and preferably with a living maternal grandfather, were identified from a register of DMD families in Wales, supplemented by families from other centres. Only those families in which the diagnosis was fully documented or which could be confirmed clinically were studied. Key obligatory carriers were first identified and blood samples taken. DNA was prepared, digested with *TaqI* and hybridized with radioactive λ RC8 probe to determine which key carriers were heterozygous for the restriction fragment length polymorphism. If the key carrier was heterozygous, samples were collected from all other available informative members of the family. Sampling of female members was restricted to obligatory carriers; those females considered carriers on the sole basis of biochemical assay (serum creatine kinase) were excluded.

DNA from 45 unrelated obligatory carriers for Duchenne was digested with *TaqI* and screened with λ RC8; nine were heterozygous for the polymorphism, giving gene frequencies in the Duchenne carriers of 0.89 for allele 1 and 0.11 for allele 2. It is thus present at the same frequency in DMD families as

in the control London population. The *TaqI* polymorphism was traced through nine families (Fig. 3) and is inherited in a mendelian fashion. Of 24 meioses, 21 retained linkage and 3 showed recombination.

Lod scores for the linkage between the polymorphism and the DMD locus (Table 1) were calculated using the LIPED computer program¹⁸ and checked by hand. The overall probability of linkage is equivalent to the area beneath the curve of likelihood values at different genetic distances.

A lod score expresses in logarithmic form the probability that a linkage exists between two loci; for DMD and λ RC8, the probability of linkage at a distance of ~ 10 centimorgans is 58:1 (making the assumption that linkage exists). The overall probability of any linkage is 22:1. The use of lod scores in this way is explained in detail by Race and Sanger¹⁹. From our experience, an analysis of at least 50 more obligatory carriers from well characterized DMD families would provide a further 10 informative pedigrees to confirm this assignment absolutely; therefore, we are making λ RC8 available to others with access to DMD families.

DMD shows negative lod scores with colour blindness (Table 1). This is to be expected because colour blindness is within the *G6PD* linkage group on the end of the long arm of the X chromosome (Xq28)²⁰. It provides a negative control and demonstrates the difference in lod scores between unlinked (but syntenic) and loosely linked loci. Using the Xg blood group, we have not yet studied sufficient informative families for λ RC8 to obtain significant results, but clearly we have confirmed that there is no measurable linkage between Xg and DMD, thus confirming the exclusion of the terminal portion of the short arm of the X chromosome as the site of the DMD locus.

The results obtained suggest that the Duchenne locus is localized approximately in the middle of the short arm of the X chromosome. This supports data from six female cases of Duchenne muscular dystrophy with balanced *de novo* X-chromosome to autosome translocations with break points at Xp21 (refs 21, 22). As these data depend on an unequivocal clinical diagnosis of DMD in females, they have hitherto been regarded as highly suggestive rather than conclusive.

In this analysis, we have assumed that DMD is a single gene defect. As in the case of the β -globin gene locus in β -thalassaemia, it is possible that only a single locus is involved, but with several different molecular defects at that gene.

These data support the localization of the gene mutation causing Duchenne muscular dystrophy to the short arm of the X chromosome, and provide an example of how to use recombinant DNA technology to define linkage to a phenotype caused

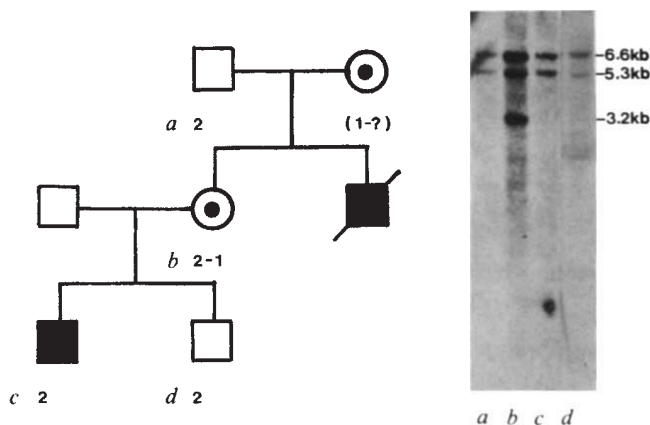


Fig. 3 Segregation of the λ RC8 polymorphism through a DMD family showing the mendelian inheritance of the variant and also a cross-over between the two loci. Nine families were studied; full details are available from the authors. Symbols: square, male; black square, affected male; circle, female; dotted circle, obligatory carrier; oblique line through symbol, dead. The autoradiograph shows the hybridization of λ RC8 to *TaqI*-digested DNA from members of the pedigree: a, grandfather; b, mother; c, d, sons.

by a single gene defect where the primary biochemical defect is unknown.

This work was supported by grants from the Cystic Fibrosis Research Trust, the MRC, the Muscular Dystrophy Group and a grant from the Deutsche Forschungsgemeinschaft to H. H. Ropers. We thank Hans Hilger Ropers and Peter Goodfellow for providing hybrid cell lines, David Hartley, Pauline Taylor and Rob Elles for probe characterization, Dr Mansoor Sarfarazi for LIPED computer analysis and Dr Tom O'Brien for help with sample collection and study of the families, Dr Ruth Sanger and Dr Patricia Tippet for performing the Xg analysis on DMD families, and the patients and their families for their cooperation; also the numerous clinicians who allowed us access to the patients under their care.

Received 5 July; accepted 23 September 1982.

- Emery, A. E. H. in *Pathogenesis of Human Muscular Dystrophies* (ed. Rowland, L. P.) 42-52 (Excerpta Medica, Amsterdam, 1977).
- Harper, P. S. in *Diseases of the Motor Unit* (ed. Schotland, D. L.) 821-844 (Wiley, New York, 1982).
- Blyth, H. et al. *J. med. Genet.* **2**, 157-160 (1965).
- Emery, A. E. H. *J. med. Genet.* **3**, 92-95 (1966).
- Little, P. F. R. et al. *Nature* **285**, 144-147 (1980).
- Little, P. F. R. in *Genetic Engineering* Vol. 1 (ed. Williamson, R.) 61-102 (Academic, London, 1981).
- Hill, M. E., Davies, K. E., Harper, P. & Williamson, R. *Hum. Genet.* **60**, 222-226 (1982).
- Orkin, S. H. et al. *New Engl. J. Med.* **299**, 166-172 (1982).
- Jeffreys, A. J. *Cell* **18**, 1-10 (1979).
- Solomon, E. & Bodmer, W. F. *Lancet* **i**, 923 (1979).
- Botstein, D., White, R. L., Skolnick, M. & Davis, R. W. *Am. J. hum. Genet.* **32**, 314-331 (1980).
- Ruddle, F. H. *Nature* **294**, 115-120 (1981).
- Dahl, H. H., Flavell, R. A. & Grosfeld, F. G. in *Genetic Engineering* Vol. 2 (ed. Williamson, R.) 50-129 (Academic, London, 1981).
- Davies, K. E., Young, B. D., Elles, R. G., Hill, M. E. & Williamson, R. *Nature* **293**, 374-376 (1981).
- Benton, W. D. & Davis, R. W. *Science* **196**, 180-182 (1977).
- Crampton, J. M., Davies, K. E. & Knapp, T. C. *Nucleic Acids Res.* **9**, 3821-3833 (1981).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Ott, J. *Am. J. hum. Genet.* **20**, 588-597 (1974).
- Race, R. R. & Sanger, R. *Blood Groups in Man* 6th edn, 551-560 (Blackwell, Oxford, 1975).
- McKusick, V. A. & Ruddle, F. H. *Science* **196**, 390-405 (1977).
- Lindenbaum, R. H., Clarke, G., Patel, C., Moncrieff, M. & Hughes, J. T. *J. med. Genet.* **16**, 389-392 (1979).
- Zatz, M., Vianna-Morgante, A. M., Campos, P. & Diamant, A. J. *J. med. Genet.* **18**, 442-447 (1981).
- Jeffreys, A. J. & Flavell, R. A. *Cell* **12**, 1097-1108 (1977).
- Kan, Y. W. & Dozy, A. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5631-5635 (1978).

Anti-ovalbumin monoclonal antibodies interact with their antigen in internal membranes of *Xenopus* oocytes

G. Valle, E. A. Jones & A. Colman

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

The microinjection of selected antibodies into the cytosol or nucleus of living cells is proving a useful technique for investigating the function of intracellular proteins¹⁻⁵. The rationale underlying this approach is that, in binding to its antigenic determinant, the antibody may mask or disrupt the activity mediated by the region of the protein near the determinant. This technique, however, cannot be used to identify functions related to antigenic determinants of proteins within the lumen of the endoplasmic reticulum (ER), or Golgi compartments, since both regions are inaccessible to direct microinjection and since the movement of proteins into these areas from the cytosol occurs during but not after their translation⁶. In theory, this constraint could be overcome if the mRNAs specifying the antibody were injected into the cell containing the target antigenic determinant. After the vectorial and co-translational transfer of its constituent chains through the membrane, the antibody would then be assembled within the lumen of the ER thereby being in a position to bind to its antigenic determinants. We demonstrate here a successful example of such an approach.

To study the interaction of antibody and antigen we made use of *Xenopus* oocytes. These cells will faithfully export secre-

tory proteins after injection of the encoding mRNAs^{7,8}. Additionally both assembly and secretion of a mouse immunoglobulin, MOPC 21, have been shown to occur normally in oocytes⁹. However, since the antigens recognized by this antibody are unknown, the biological activity of the oocyte product remains untested. Moreover, the oocyte might not possess the high levels of disulphide interchange enzyme thought to be necessary in immunoglobulin-producing cells for the production of correctly located S-S bonds within the immunoglobulin molecule¹⁰. In this letter we describe the synthesis in oocytes of murine monoclonal antibodies raised against chick ovalbumin, a protein whose synthesis and secretion from oocytes has been well characterized^{11,12}. The antibodies synthesized by the oocyte maintain their specificity. When ovalbumin mRNA is also injected the formation of antigen-antibody complexes within the internal membranes of the oocyte results in a severe reduction in secreted antibody, even when the ovalbumin mRNA was introduced 24 h later.

Spleen cells from a BALB/c mouse immunized with ovalbumin were fused to P3-NS1 myeloma cells in the presence of polyethylene glycol¹³. The fusion was screened by an indirect trace binding assay¹⁴ and positive wells cloned by the dilution method¹⁵. Two cloned hybridomas 7/2 and 3/4, both IgG₁, were chosen for the microinjection studies. Binding studies carried out under near-saturating conditions for both monoclonals and detecting antibody suggested that their binding was additive, indicating that they recognized different antigenic determinants on the ovalbumin molecule (data not shown). Furthermore, the stoichiometry suggested single antigenic sites on the molecule.

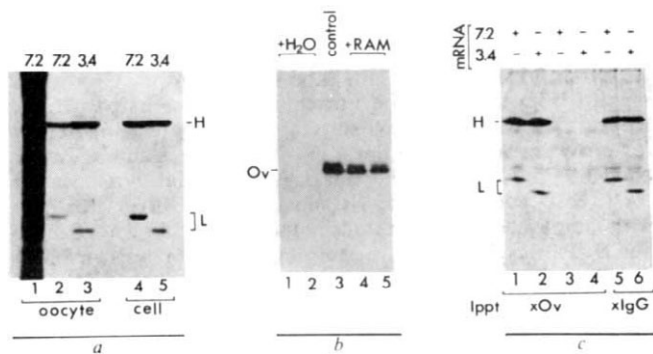
Immunoprecipitates of the immunoglobulin chains synthesized by the hybridoma cells had the same electrophoretic mobility as the analogous immunoprecipitated products made in oocytes after injection of the hybridoma mRNAs (Fig. 1a). Chick ovalbumin made by oocytes differs slightly in its post-translational modification from the oviduct protein¹¹. Nevertheless the immunoglobulins made by the hybridoma cells bind to ovalbumin synthesized by oocytes after injection of oviduct mRNA (Fig. 1b).

Since the oligosaccharide moiety elaborated by oocytes for the glycosylation of immunoglobulins (A.C. and G.V., unpublished data) and other foreign proteins¹⁶ differs from the 'native' side-chain, it was possible that the immunoglobulins synthesized by the oocyte would lack biological specificity. However, the monoclonal antibodies translated by the oocytes bound specifically to an ovalbumin immunoabsorbant (Fig. 1c).

The data above show that the monoclonal antibodies translated by oocytes and subsequently extracted will bind to ovalbumin. However, antibody conformation is dependent on correct disulphide bond formation. Since the exact redox potential within the ER of cells is unknown, and since disulphide bonds in immunoglobulins might be somewhat labile within the ER due to the presence of a disulphide-exchange enzyme¹⁰, the biological activity manifested by the immunoglobulins after their extraction from oocytes might not exist in the intracellular environment. In order to examine this possibility we have injected various combinations of oviduct and hybridoma mRNAs into oocytes. The oocytes were incubated in medium containing ³⁵S-methionine for 24 h before oocytes and media were collected for analysis. Oocyte homogenates and media were immunoprecipitated with rabbit anti-chick ovalbumin or goat anti-mouse immunoglobulin antibodies, and the resulting immunoprecipitates examined by polyacrylamide gel electrophoresis (Fig. 2).

When the oocytes were injected with ovalbumin mRNA and both monoclonal mRNAs, the monoclonal antibody chains could be specifically precipitated by rabbit anti-ovalbumin (Fig. 2, track 3). Similarly, from the same oocytes, ovalbumin could be specifically precipitated by goat anti-mouse immunoglobulin (Fig. 2b, tracks 3 and 9). However, if only one monoclonal mRNA was co-injected with ovalbumin mRNA, this cross-precipitation effect was never observed although some non-

Fig. 1 Biological specificity of antibodies made by oocytes. The 3/4 and 7/2 lines were grown in Dulbecco's modified Eagle's medium containing 10% horse serum (Difco) supplemented with hypoxanthine, aminopterin and thymidine. Cells were labelled with ^{35}S -methionine (Amersham) as described by Cotton *et al.*²². Media and cell homogenates were retained for immunoprecipitation and electrophoresis. Hybridoma mRNA was prepared by lysing 5×10^9 cells in hypotonic buffer containing 0.1% diethylpyrocarbonate (Sigma). The post-nuclear supernatant was twice phenol-extracted and then ethanol precipitated. The RNA pellet was resuspended in 5 ml of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS and 0.5 M LiCl, bound to oligo (dT) cellulose (Type III, Collaborative Research) and eluted with the same buffer minus LiCl. The polyadenylated mRNA was then ethanol precipitated and resuspended in distilled water at 1 mg ml^{-1} . Polyadenylated oviduct mRNA was prepared from laying Rhode Island Red hens as described by Palmiter²³. For clarity this mRNA is referred to as ovalbumin mRNA in the text. Oocytes were microinjected with 30 nl of mRNA and cultured in Barth's saline (5 oocytes/30 μl) containing antibiotics, 6% fetal calf serum and $1 \mu\text{Ci } \mu\text{l}^{-1}$ ^{35}S -methionine (300 Ci mmol^{-1}) for 24 h at 21°C . Culture media were retained for electrophoresis whilst oocytes were homogenized in 100 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulphonyl fluoride, 25 mM Tris pH 7.6 as previously described⁷. For electrophoresis the cell lysates, oocyte fractions and culture media were immunoprecipitated (see below). Precipitates were dissolved in sample buffer (20 mM Tris-HCl pH 9.2, 20% glycerol, 1 mM phenylmethylsulphonyl fluoride, 1% β -mercaptoethanol, 0.01% bromophenol blue and 2% SDS) and 25 μl aliquots were run on 12.5% polyacrylamide gels, followed by fluorography²⁴. Numbers above tracks refer to mRNA injected (oocyte) or cell lines used (cells). *a*, Synthesis and secretion of immunoglobulins by cells and oocytes. 50 μl aliquots of homogenates from mRNA-injected oocytes (tracks 2, 3) or labelled cells (tracks 4 and 5) were immunoprecipitated with 3 μl of goat anti-mouse IgG (Miles) using *Staphylococcus aureus* envelopes as previously described⁹. Track 1 contains 5 μl of oocyte homogenate before immunoprecipitation. *b*, Ovalbumin made by oocytes binds to hybridoma antibodies. Oocytes were injected with ovalbumin mRNA, labelled with ^{35}S -methionine and homogenized as described above. 50 μl of homogenate were mixed with 500 μl of immunoprecipitation buffer⁹ and with 50 μl of hybridoma cell medium containing the monoclonal antibodies. The above mixture was incubated for 1 h on ice. Then either 10 μl of rabbit anti-mouse IgG (RAM) (tracks 4 and 5) or 10 μl of distilled water (tracks 1 and 2) were added and the incubation was continued for another 30 min on ice. Finally 50 μl of *S. aureus* suspension were added. The immunoprecipitation was completed as usual and samples run into a gel as described above and fluorographed. Track 1, 3/4 medium without RAM; track 2, 7/2 medium without RAM; track 3, control immunoprecipitated with rabbit anti-ovalbumin; track 4, 3/4 medium plus RAM; track 5, 7/2 medium plus RAM. Ov, ovalbumin. *c*, Oocyte anti-ovalbumin immunoglobulins bind to ovalbumin. 50 μl aliquots of 10% *S. aureus* suspension in immunoprecipitation buffer were incubated with 3 μl of rabbit anti-ovalbumin (Miles; tracks 1–4) or with 3 μl of goat anti-mouse IgG (tracks 5, 6), for 30 min at room temperature in order to bind the antibodies to the bacterial envelopes. The *S. aureus* envelopes were then spun and rinsed twice with 500 μl of immunoprecipitation buffer, resuspended in 500 μl of the same buffer and incubated for 30 min in presence (tracks 1, 2 and 5, 6) or absence (tracks 3, 4) of 0.2 mg ml^{-1} ovalbumin (Sigma). The envelopes were then rinsed twice as above and resuspended in 500 μl of immunoprecipitation buffer. Meanwhile, homogenates were made from oocytes previously microinjected with 7/2 mRNA or 3/4 mRNA and subsequently labelled as in Fig. 2a. 10 μl of oocyte homogenate were added to the appropriately prepared, resuspended envelopes and incubated overnight at 4°C . The rest of the immunoprecipitation and the electrophoresis were carried out in the standard way described above. Ov, ovalbumin; H, immunoglobulin heavy chain; L, immunoglobulin light chain; Ippt, immunoprecipitation with rabbit anti-chick ovalbumin (xOv) or goat anti-mouse IgG (xIgG).



specific precipitation of ovalbumin occurred which disappeared if unlabelled ovalbumin was included in the homogenization medium (compare Fig. 2b, tracks 2–4 with tracks 8–10). This complex was not found when oocytes containing the two monoclonal antibodies and oocytes containing ovalbumin were mixed before homogenization (data not shown). We therefore interpret the co-precipitation of ovalbumin and the two monoclonal antibodies as indicating their association *in vivo* within the membranous systems of the oocyte.

Figure 2 shows that the two monoclonal antibodies were also found associated with secreted ovalbumin present in the culture media (Fig. 2a, track 11; Fig. 2b, track 13). However, control experiments revealed that such association could take place in the media after secretion (data not shown) so it is not clear whether the monoclonal antibody–ovalbumin complex can be secreted.

The effect on secretion resulting from interactions between the immunoglobulins and ovalbumin was assessed by comparing the relative amounts of each protein inside and outside the oocyte under the different injection regimes. The results of some representative experiments are shown in Fig. 3a. Only ovalbumin and the two immunoglobulin γ chains were considered since excess light (κ) chains are made and secreted by the oocytes (data not shown); these supernumerary chains will not be present in complexes between ovalbumin and tetrameric immunoglobulin. The results in Fig. 3a clearly show that not only was there a marked reduction of heavy chain secretion when both monoclonal antibodies were present with ovalbumin, but also when each immunoglobulin alone was present with ovalbumin. This latter result was surprising in view of our failure to detect any specific interactions by immunoprecipitation in these cases (see Fig. 2).

No significant reduction of ovalbumin secretion was seen or expected in these experiments, since only a small proportion of ovalbumin was bound to immunoglobulin inside the oocytes (see Fig. 3c).

We have recently demonstrated that MOPC 21 heavy (H) chain trapped in oocytes due to the absence of the complemen-

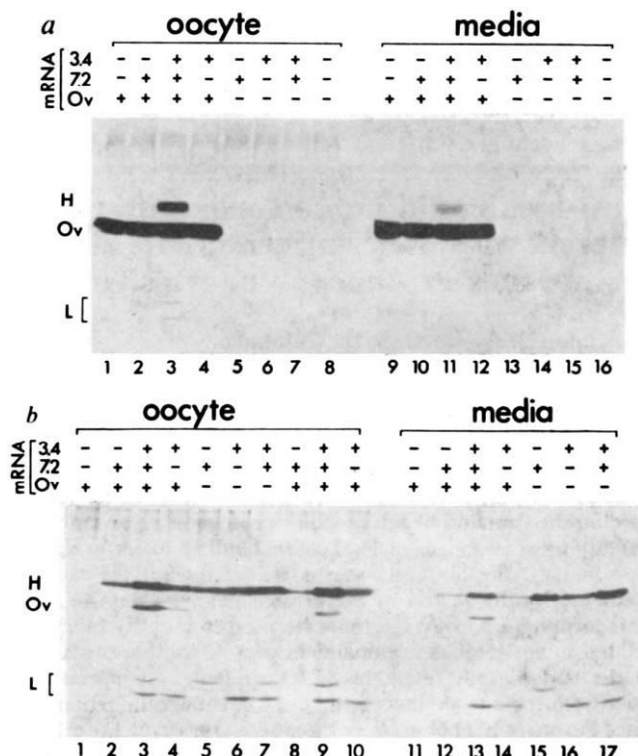


Fig. 2 Coinjection of ovalbumin and anti-ovalbumin mRNAs into oocytes. 7/2 mRNA (0.3 mg ml^{-1}), 3/4 mRNA (0.3 mg ml^{-1}) and ovalbumin mRNA (0.04 mg ml^{-1}) were injected as indicated above into oocytes. Oocytes were cultured as described in Fig. 1 and processed for immunoprecipitation with rabbit anti-ovalbumin (*a*) or goat anti-mouse (IgG) (*b*) antibodies. In *b* tracks 8–10 homogenization and immunoprecipitation media were supplemented by the addition of unlabelled ovalbumin to 1 mg ml^{-1} final concentration. Immunoprecipitates were electrophoresed on 12.5% PAGE gels under reducing conditions and then fluorographed. Ov, ovalbumin; H, immunoglobulin heavy chain; L, immunoglobulin light chain.

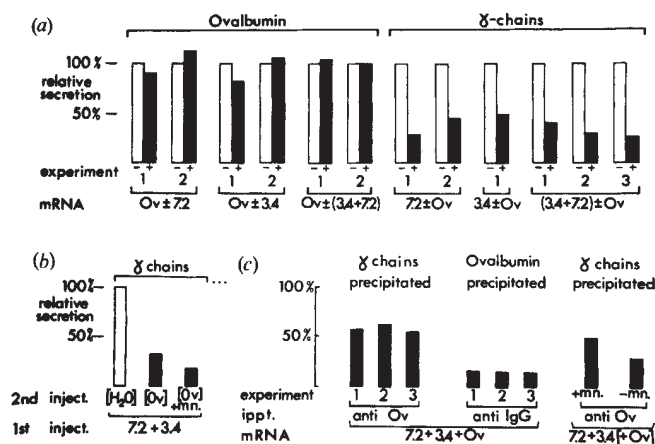


Fig. 3 Quantification of the interactions of monoclonal antibodies with ovalbumin. *a*, Comparisons of the ratios of secreted to intracellular ovalbumin or immunoglobulin γ chains. Appropriate bands from the gels shown in Fig. 2 and other similar experiments were excised and counted in toluene-based scintillant. Corrections were made for radioactivity migrating in the corresponding regions of control tracks. The ratios of secreted to intracellular protein were calculated, converted to percentages and adjusted to 100% for the controls (open blocks) where only one mRNA class (hybridoma or oviduct) was injected. Stippled blocks indicate the injection of both mRNA classes. *b*, Staggered mRNA injection. Appropriate bands from the gel shown in Fig. 4 were treated as above. The mRNAs introduced in the second injection are shown in square brackets. mn, Monensin. *c*, Degree of co-precipitation. The amounts of intracellular ovalbumin or immunoglobulin γ -chain immunoprecipitating with each antibody was calculated by excising gel bands from tracks corresponding to oocyte samples where ovalbumin mRNA, 7/2 mRNA and 3/4 mRNA had been co-injected (for example, Fig. 3, tracks 3; Fig. 5b, tracks 12, 13). The amount of the γ chains or ovalbumin which co-precipitated with rabbit anti-ovalbumin or goat anti-mouse IgG respectively is expressed as % of the total amount of each protein directly precipitated by its antibody. The two blocks on the right of this figure correspond to the staggered injection experiment of Fig. 4 with the second mRNA shown in square brackets. mn, Monensin; ippt, immunoprecipitating antibody. When the immunoglobulin tetramers were excised from a non-reducing gel and re-electrophoresed under reducing conditions, band analysis revealed that the heavy chains contained 4–5 times more radioactivity than the light chains. This indicates that the γ -chain contains 4 or 5 methionines or multiples thereof. Using this information and the calculated radioactive contents of γ -chain and ovalbumin (16 methionines) bands in the experiments above, we estimate there to be 1.5 times (or multiples thereof) more ovalbumin than γ -chain in the oocytes.

tary light (L) chain can be rescued by assembly into an H₂L₂ tetramer as a result of an injection of light chain mRNA 24 h later¹⁷. We were therefore interested to see whether any of the radioactive immunoglobulin molecules remaining in injected oocytes 12 h after the imposition of unlabelled methionine 'chase' conditions were accessible to ovalbumin molecules translated from a post-chase injection of oviduct mRNA. Figure 4 illustrates such a staggered injection experiment. Quantitation of the results shown in Fig. 4 demonstrates first that immunoglobulin secretion was reduced by 60% in the presence of ovalbumin (Fig. 3b) and second that when homogenates of such double-injected oocytes were precipitated with rabbit anti-ovalbumin, 29% of the intracellular γ chain co-precipitated, a somewhat lower proportion than the 60% found when the two mRNAs were co-injected (Fig. 3c). This value increased to 49% if secretion was further inhibited (Fig. 4, tracks 5 and 6) by use of the ionophore monensin, an agent known to inhibit secretion^{18,19} by disrupting the Golgi apparatus²⁰.

The anti-ovalbumin monoclonal antibodies synthesized by oocytes are clearly specific for ovalbumin after their individual extraction from oocytes. In contrast, intracellular interaction, as identified by immunoprecipitation, between the monoclonal antibodies and ovalbumin can only be shown when both antibodies are present. This might be a consequence of the low affinity of these particular antibodies for their antigenic determinants. Since the two antibodies recognize different determinants an aggregate between ovalbumin and the antibodies is expected. The formation of an aggregate might consolidate the weak interactions between individual molecules²¹. Under

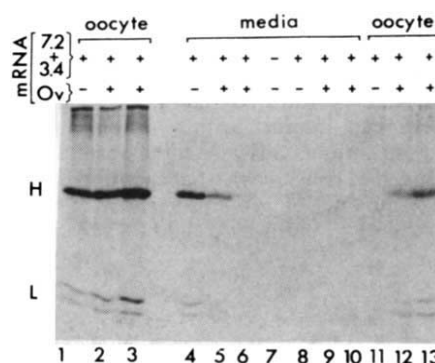


Fig. 4 Staggered injection of ovalbumin and anti-ovalbumin mRNAs. Oocytes were injected with 7/2+3/4 mRNA (both at 0.3 mg ml⁻¹) and immediately cultured overnight in media containing ³⁵S-methionine with (tracks 3, 6, 10, 13) or without 30 μ M monensin (Calbiochem). Healthy oocytes were transferred to unlabelled media supplemented with 10 mM methionine for a further 12 h. Ovalbumin mRNA (1 mg ml⁻¹) was then injected into some oocytes and incubation continued for 24 h. Oocytes and media were immunoprecipitated with goat anti-mouse IgG (tracks 1–7) or rabbit anti-ovalbumin (tracks 8–13) antisera and electrophoresed on a 12.5% gel. A 4-day fluorogram is shown. Ov, ovalbumin; H, immunoglobulin heavy chain; L, immunoglobulin light chain.

these circumstances secretion of the tetrameric immunoglobulin was significantly reduced. This is not particularly surprising considering the potentially large size of the aggregate. What is surprising, however, is the reduction of immunoglobulin secretion when only one antibody is present. This reduction is not due to competition with the ovalbumin for some limiting oocyte component necessary for translocation since we have previously demonstrated that mRNA transition itself is the only process which limits the export of secretory proteins from oocytes¹¹. Additionally, secretion from oocytes of a murine anti-membrane protein antibody was unaltered by the presence of ovalbumin (Fig. 5). More likely, the reduction of secretion is due to the existence of an equilibrium between slow or unmovable antibody-(ovalbumin)₂ trimers and the individual molecules. Even so it is unclear why the movement of such trimers would be reduced unless a conformational change induced by binding in either antigen or antibody is incompatible with the secretory mechanism.

The demonstration that ovalbumin introduced via mRNA injection into oocytes already containing the anti-ovalbumin immunoglobulins can associate with some of the intracellular immunoglobulin indicates that secretory proteins whose

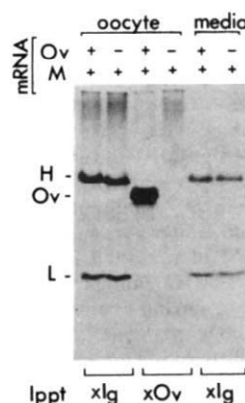


Fig. 5 Secretion of ovalbumin and anti-*Xenopus* immunoglobulins from oocytes. Total RNA was prepared from the murine hybridoma line 18H-11 as described in Fig. 1. This hybridoma secreted an antibody which reacts with a *Xenopus* membrane antigen (E. Jones, unpublished). The RNA at 2 mg ml⁻¹ and ovalbumin mRNA (0.06 mg ml⁻¹) were injected into oocytes and the culture, processing of oocytes and media, and electrophoresis as in Fig. 2. Ov, ovalbumin; M, RNA from 18H-11; H, immunoglobulin heavy chain; L, immunoglobulin light chain; Ippt, immunoprecipitation with rabbit anti-chick ovalbumin (xOv); goat anti-mouse IgG (xIg).

synthesis is temporally separated, can nevertheless become spatially apposed. However, the intracellular location for this interaction remains undetermined.

Thus we have demonstrated that monoclonal antibodies and their antigens can interact within the ER of the oocyte. Evidently these interactions affect the resultant secretion of these proteins. It is conceivable that the function of lumenally-exposed determinants of proteins resident along, or passing through the secretory pathway could be gauged by the disruption

of effects of introducing monoclonal antibodies raised against them. There seems no reason why this approach cannot be extended to microinjection of cultured cells where the possibilities of combining immunofluorescent and biochemical techniques would facilitate analysis.

We thank Daniel Cutler for the gift of ovalbumin mRNA, and S. Bhamra and A. Rughani for technical assistance. This research was supported by the Cancer Research Campaign (A.C. and G.V.) and the MRC (E.J.).

Received 9 July; accepted 1 September 1982.

1. Antman, K. H. & Livingston, D. M. *Cell* **19**, 627–635 (1980).
2. Bona, M., Scheer, U. & Bautz, E. K. F. *J. molec. Biol.* **151**, 81–99 (1981).
3. Lin, J. & Feramisco, J. R. *J. Cell Biol.* **87**, 181a (1980).
4. Rungger, D., Rungger-Brandle, E., Chaponnier, C. & Gabbiani, G. *Nature* **282**, 320–321 (1979).
5. Wehland, J., Willingham, M., Dickson, R. & Pastan, I. *Cell* **25**, 105–119 (1981).
6. Sabatini, D. D., Kreibichg Mortimoto, T. & Adesnik, M. *J. Cell Biol.* **92**, 1–22 (1982).
7. Colman, A. & Morser, J. *Cell* **17**, 517–526 (1979).
8. Lane, C. *et al. Eur. J. Biochem.* **111**, 225–235 (1980).
9. Valle, G., Besley, J. & Colman, A. *Nature* **291**, 338–340 (1981).
10. Roth, R. A. & Koshland, M. E. *Biochemistry* **20**, 6594–6599 (1981).
11. Colman, A. *et al. Eur. J. Biochem.* **113**, 339–348 (1981).
12. Cutler, D., Lane, C. & Colman, A. *J. molec. Biol.* **153**, 917–931 (1981).

13. Kohler, G. & Milstein, C. *Nature* **256**, 495–497 (1975).
14. Williams, A. F., Galfre, G. & Milstein, C. *Cell* **12**, 663–673 (1974).
15. McKearn, T. J. in *Monoclonal Antibodies* (eds Kennet, R. H., McKearn, T. J. & Bechtol, K. B.) 374 (Plenum, New York, 1980).
16. Mous, J., Peeters, B. & Rombauts, W. *FEBS Lett.* **122**, 105–108 (1980).
17. Colman, A., Besley, J. & Valle, G. *J. molec. Biol.* (in the press).
18. Tartakoff, A. & Vassalli, P. *J. Cell Biol.* **79**, 694–707 (1978).
19. Tartakoff, A. & Vassalli, P. *J. Cell Biol.* **83**, 284–299 (1979).
20. Somlyo, A. P., Garfield, R., Chacko, S. & Somlyo, A. V. *J. Cell Biol.* **66**, 425–443 (1975).
21. Ehrlich, P. H., Moyle, W., Moustafa, Z. A. & Canfield, R. E. *J. Immun.* **128**, 2709–2713 (1982).
22. Cotton, R. G. H., Secher, D. S. & Milstein, C. *Eur. J. Immun.* **3**, 135–140 (1973).
23. Palmiter, R. D. *J. biol. Chem.* **248**, 2095–2106 (1973).
24. Bonner, W. & Laskey, R. *Eur. J. Biochem.* **46**, 83–88 (1974).

A cDNA sequence encoding a rabbit heavy chain variable region of the V_{H2} allotype showing homologies with human heavy chain sequences

K. E. Bernstein, E. Premkumar Reddy*,
C. B. Alexander & R. G. Mage

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, and *Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, USA

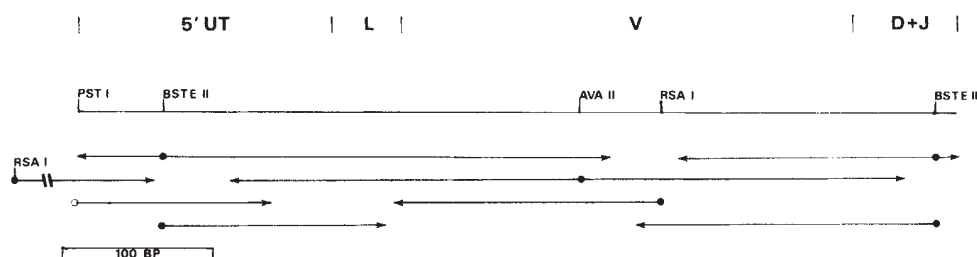
There are only four positions in the amino acid sequences of immunoglobulin variable region domains of all species at which the same amino acid is always found¹. The compiled sequences of rabbit heavy chain variable (V_H) domains have 48 invariant positions^{1,2} and at 14 additional positions in the first and third framework segments the different amino acids found correlate with the rabbits' V_{H1} allotype^{3–5}. This V_H allotypic system with three common 'alleles' V_{H1} , V_{H2} and V_{H3} inherited in simple mendelian fashion^{3,4} poses the question of how gene families with distinct sets of conserved codons have evolved and been maintained in the midst of information leading to hypervariability. Here we report a cDNA sequence that encodes a protein characteristic of the rabbit V_{H2} allotype and we predict the allotype-associated codons that may be found in V_{H1} and V_{H3} genes. We have also found within the second hypervariable, complementarity-determining region (CDR2) encoded by our cDNA clone, a DNA sequence highly homologous to a human D_H minigene⁶. This observation and a similar one of Wu and Kabat⁷, who identified a D_H minigene sequence in the CDR2 of a cloned human V_H gene⁸, lends support to their idea that D minigene information has contributed to CDR2 either during evolution or ontogeny⁷. Gene conversion^{9,10}, minigene assortment^{11,12} or insertion¹³ mechanisms may be involved both in the generation of hypervariability and the conservation of allotype codons.

A partial cDNA library from splenic mRNA of V_{H2} rabbits infected with *Trypanosoma equiperdum* was the source of clone $\mu 3$. This cDNA clone is a full-length copy of mRNA encoding the hydrophobic leader, V_H and C_H regions¹⁴. DNA was sequenced by the procedures of Maxam and Gilbert¹⁵. The variable region restriction map and our sequencing strategy are shown in Fig. 1. Figure 2a shows the nucleotide sequence and the deduced amino acid sequence of the variable region of $\mu 3$.

There are no rabbit nucleic acid sequences of cDNAs or genomic V_H , D_H or J_H genes with which to compare our sequence, but we found considerable homology with a cloned human genomic V_H segment, $V_H 26$ (ref. 8) and with the human $J_H 4$ minigene segment¹⁶ and show these for comparison in Fig. 2a. Different genomic sequences for human J segments encode 15–17 amino acids and through comparison with human $J_H 4$ it is probable that the Phe (TTC) at amino acid position 100C of $\mu 3$ was contributed by a codon from a J minigene. The Phe (TTT) preceding it could have been D - or J -encoded or the site of intracodon recombination between D and J . The D portion of $\mu 3$ appears to encode seven or eight amino acids (positions 95–100A or 100B) and the sequence encoded bears no resemblance to any published rabbit protein sequences in this region^{1,17}. The nucleic acid sequence of our rabbit V_H (Fig. 2a) is remarkably homologous to the human $V_H 26$ sequence (73% in the leader and V_H amino acid positions –20 to +94) and to the human $J_H 4$ minigene (80% homology for amino acid positions 100C–113). A striking and unexpected degree of homology is found in the CDR2 of these two sequences. Introduction of a codon gap at amino acid position 52 and deletion of two codons at positions 55 and 56 in the rabbit sequence allows alignment of 17 of 18 bases within this 'hyper-variable region'. This is precisely the part of the human CDR2 (amino acid positions 52A–56) in which Wu and Kabat found a sequence of 14 nucleotides homologous to the human $D2$ minigene⁷. These 14 nucleotides are enclosed in a box in Fig. 2a. As shown in Fig. 2b, we find a similar homology although to a lesser degree when the rabbit sequence between the codons for amino acids 49 and 59 is compared with human $V_H 26$ and the human $D1$ and $D2$ minigene sequences⁶. These observations lead one to ask how it is that a sequence homologous to a D minigene is found in the CDR2 of both rabbit and human V_H regions. In mice the hypervariability of the third CDR results from rearrangements that join DNA segments coding for V_H , D_H and J_H during B lymphocyte differentiation^{18,19}. The genetic basis of hypervariability of the first and second complementarity determining regions (CDR1 and CDR2) is less well understood. Wu and Kabat have suggested an insertion mechanism for the generation of hypervariable region diversity¹³. Indeed, the portion of CDR3 encoded by one or more D_H segments of mice is such an insert^{18,19}. The present observations lend further but indirect support to the idea that D minigene information has somehow contributed to the sequence of the second CDR either through mechanisms of gene conversion^{9,10}, minigene assortment^{11,12} or insertion¹³.

The number of J_H genes present in the rabbit genome is unknown. However, published protein sequences that include the segment presumed to be encoded by the J_H segment show

Fig. 1 Strategy used in sequencing the DNA of clone μ 3. The top of the figure indicates the relative sizes of the 5' untranslated region (5' UT), the leader sequence (L), the variable region proper (V) and the D and J segments. All sequencing was done using the method of Maxam and Gilbert¹⁵. Solid and open circles indicate fragments 5' or 3' labelled, respectively. The arrows and lines show the length and direction of sequencing. The *Rsa*I site in pBR322 at position 3,847 was also used. The relative size of 100 base pairs (bp) is indicated.



little deviation from a prototype sequence between positions 101 and 113^{2,17}. Clone μ 3 encodes the 11 invariant amino acids at positions 103–113 but has Ala replacing Asp and Ile replacing Val at 101 and 102. Whether this is due to a different germ-line *J* segment, somatic mutation or some other mechanism is unknown. The usual Asp and Val can be arrived at by single base changes from the GCC and ATC we observed in clone μ 3.

In our V_H sequence, unusual amino acids are found at positions 93 and 94. This is a highly conserved area of the V_H region and in all other published sequences of rabbit V_H , the amino acids at positions 93 and 94 have been alanine and arginine. Clone μ 3 encodes Gly and Ser at these positions; the sequence was confirmed on both strands. The Gly and Ser codons (GGG, AGT) are potentially derived by single base changes from codons for Ala (GCG) and Arg (CGT). According to Kabat *et al.*¹, of 73 published sequences of V_H regions from all species, 64 have Ala at position 93; Gly has not been reported before. Similarly, 54 of 73 V_H sequences have Arg at position 94 with one reported Ser.

With the exception of the Ala and Arg at 93 and 94 and a deletion at position 2 that is found in 12 of 14 rabbit V_H sequences, each of the 33 other invariant residues listed by Kabat *et al.*¹ are encoded by μ 3. The same pattern is found when considering the amino acids that are thought to comprise the V_H allotypes. Although relatively few rabbit V_H proteins have been sequenced, sequence comparisons suggest that the amino acids determining a particular V_H allotype are clustered in FR1 and FR3 (positions 5–17 and 65–85). Figure 3a summarizes the positions at which V_H allotype-associated amino acids occur. Comparison of our deduced amino acid sequence for these positions with known V_H protein sequences clearly shows

that clone μ 3 encodes a V_H sequence characteristic of the a2 allotype. As shown in Fig. 3b, with two exceptions (a2 Asp to a1 Thr at position 16 and a2 Gly at position 84 to a1 Thr), the codons of μ 3 found at allotype-associated positions can theoretically be changed by one base to give a codon for the amino acids typical of the a1 and a3 allotypes. The codons predicted to occur in a1 and a3 are also compared with those found in the human V_H26 gene (Fig. 3b). The codons of V_H26 match the predicted codons at 6 of the 14 positions compared and two additional codons differ only in the third base. Although there have been no confirmed reports of V_H -like alleles in man, some rabbit anti-a1 allotype antibodies do react with some human immunoglobulins but do not detect a polymorphic system²⁰. The present comparison of the allotypic regions of rabbit with human V_H suggests that 'allelic' variants could occur in man.

It has been suggested that rabbits of different 'allelic' V_H phenotypes share the same set of coding sequences but have different allelic regulatory elements²¹. Observations of occasional production of unexpected V_H allotypes (for example, V_H a2 in an a¹a³ rabbit²²) have supported such suggestions^{22,23}. Our data show that certain portions of the rabbit V_H sequence are highly conserved even between species. Thus a truly allotype-specific V_H probe for investigation of this problem will not be a simple one to design. Clone μ 3 constructed from mRNA of V_H a2 animals encodes a typical a2 protein sequence and thus provides a means for investigating the genetic basis of the rabbit V_H allotypes and solving the paradox of conserved V_H gene families intimately associated with mechanisms that lead to hypervariability.

We thank Myron Waxdal for help with our computer analyses, Dr T. T. Wu who provided computer printouts from his

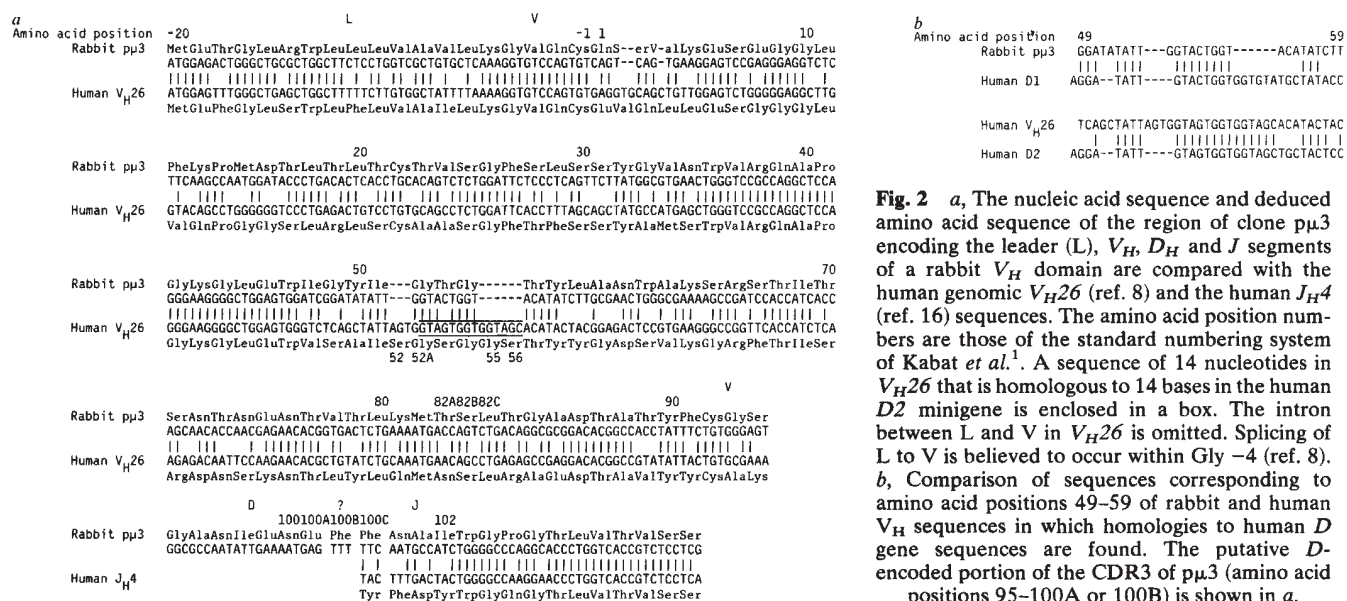


Fig. 2 *a*, The nucleic acid sequence and deduced amino acid sequence of the region of clone μ 3 encoding the leader (L), V_H , D_H and *J* segments of a rabbit V_H domain are compared with the human genomic V_H26 (ref. 8) and the human J_H4 (ref. 16) sequences. The amino acid position numbers are those of the standard numbering system of Kabat *et al.*¹. A sequence of 14 nucleotides in V_H26 that is homologous to 14 bases in the human $D2$ minigene is enclosed in a box. The intron between L and V in V_H26 is omitted. Splicing of L to V is believed to occur within Gly -4 (ref. 8). *b*, Comparison of sequences corresponding to amino acid positions 49–59 of rabbit and human V_H sequences in which homologies to human *D* gene sequences are found. The putative *D*-encoded portion of the CDR3 of μ 3 (amino acid positions 95–100A or 100B) is shown in *a*.

FR1																	FR3											
Amino acid position	5	6	7	8	9	10	11	12	13	14	15	16	17	65	66	67	68	69	70	71	72	73	74	84	85			
a2	LYSGluSerGLUGlyGlyLeuPHELYSPrometASPTHR																	SERArgSERThrIleTHRSEAsnThrAsn										GLYALA
pu3	-----																	-----										-----
K25	-----																	-----										-----
B51	-----																	-----										-----
2690	-----																	-----										-----
pool	-----																	-----										-----
a1	GLUGluSerGLYGlyARGLeuVALTHRProGlyTHRPRO																	GLYArgPHEThrIleSERLYSThrSerTHR										THRGLU
3381	-----																	-----										-----
3374	-----																	-----										-----
723569	-----																	-----										-----
3172	-----																	-----										-----
B55	-----																	-----										-----
a3	GLUGluSerGLYGlyASPLEuVALLYSPProGlyALASER																	GLYArgPHEThrIleSERLYS										ALAALA
pool	-----																	-----										-----
Human V _H 26	LEUCGluSerGLYGlyGlyLeuVALGLNProGlyGLYSER																	GLYArgPHEThrIleSERARGAspAsnSER										ALAGLU

b																
Amino acid position	5	8	10	12	13	16	17	65	67	70	71	74	84	85		
pu3 V _H a2	AAG	GAG	GGT	TTC	AAG	GAT	ACC	AGC	TCC	ACC	AGC	AAC	GGC	GCG		
V _H a1	[G--	G--	A--	G--	C--	AC--	G--	G--	T--	T--	A--	C--	AC--	A--]		
V _H a3	[G--	G--	A--	G--	---	C--	T--	G--	T--	T--	A--	C--	T--	---		
V _H 26	TT--	G--	--C	G--A	C--	--GG	T--	G--	T--	T--A	--A	TC--	T--C	FA--]		

Domain structure in high molecular weight high mobility group nonhistone chromatin proteins

Gerald R. Reeck*, Paul J. Isackson*
& David C. Teller†

* Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506, USA

† Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA

The high mobility group (HMG) proteins¹ are a small set of nonhistone chromatin proteins which exhibit preferential affinity for single-stranded DNA²⁻⁴. Large portions of the amino acid sequences of calf thymus HMG-1 and HMG-2 have recently been published⁵. We have analysed that information and describe here an internal sequence homology in the proteins which, coupled with a prediction of secondary structure, strongly suggests that all the high molecular weight HMG proteins (HMG-1, HMG-2 and HMG-E) exist in three domains of comparable lengths. The N-terminal and central domains have homologous sequences and are probably similar in conformation. The C-terminal domain is nonhomologous and highly acidic. We also present results of trypsin digestion experiments which are consistent with the postulated domain structure. We propose that the N-terminal and central domains of the proteins are DNA-binding regions and that the highly acidic C-terminal domain interacts with histones.

In our first analysis, we have used amino acid sequence comparison methods developed in one of our laboratories⁶, which we have previously used on histones⁷. The methods are an adaptation of procedures described by Sankoff⁸ for comparison of nucleic acid sequences. The Sankoff algorithm finds optimal alignments of two sequences (or families of sequences in our case) under a specified constraint on the maximum number of gaps. The statistical significance of an alignment proposed on the basis of the Sankoff algorithm is assessed by comparing the scores of the proposed alignment with the scores of alignments between pairs of randomized sequences^{7,9}. We have used the scheme of McLachlan for scoring similarities between amino acid residues¹⁰.

HMG-1 and HMG-2 have sufficiently similar amino acid sequences that an alignment between them, as previously published by Walker *et al.*⁵, is unambiguous. We therefore treat them as a two-member family^{10,11} of homologous sequences, designated HMG-1,2. We have found an internal homology in HMG-1,2 by examining the first two 100-residue portions of the family's sequence. Figure 1 shows an alignment between positions 1-92 and 98-176. The one gap is needed to optimize the alignment before and after a stretch of unknown sequence (positions 40-54). The alignment score in Fig. 1 is 1,079 in the McLachlan scoring scheme as we use it⁶. To assess the statistical significance of the alignment, the Sankoff algorithm was applied to 100 pairs of randomized sequences identical in length and amino acid composition to the sequences of positions 1-97 and 98-189. The scores of the optimized alignments (with one gap allowed) between the pairs of randomized sequences had a mean of 604 and a standard deviation of 57; the highest score observed was 723. Performing more randomizations seemed superfluous, given the large separation between the score of the proposed alignment and the scores of the alignments of randomized sequences. With the data from 100 randomizations, we are limited to concluding¹² that the level of significance of the alignment in Fig. 1 is less than 0.01. The level of significance is, in fact, much lower than 0.01. Although the distributions of scores from comparisons of randomized amino acid sequences are in general not gaussian, for relatively long sequences such as we consider here, the distribution of scores is reasonably close to gaussian⁶. Assuming a gaussian distribution, the proba-

Fig. 3 *a*, Sequences of rabbit V_H proteins of known V_H allotype are compared with the deduced amino acid sequence encoded by clone pμ3 in the parts of the first and third framework segments (FR1 and FR3) in which allotype-associated amino acids are found. The sequences are from ref. 1, in which references to the original papers are also given. The amino acids at positions implicated as V_H allotype correlates are shown in capital letters. The amino acid sequence encoded by the human V_H26 gene is also shown for comparison. Solid lines indicate identity to the a2 or a1 prototype sequences. *b*, The codons at amino acid positions implicated as V_H allotype correlates found in clone pμ3 (V_Ha2) are compared with hypothetical codons (enclosed between brackets) that would generate amino acids typically found in a1 and a3 proteins. Also compared are the homologous codons found in the human V_H26 gene sequence. Dashes indicate bases that are identical to those shown for pμ3. Boxes enclose codons in the human V_H26 sequence and hypothetical a1 and a3 sequences that are identical at all or the first two bases. A one-base change to AGC would also generate a Ser codon at position 17 of V_Ha3 sequences.

database, Shirley Starnes for typing the manuscript and our many colleagues at NIH for helpful comments. In addition we made use of The National Biomedical Research Foundation's Nucleic Acid Sequence Database and computer system. K.B. especially thanks Ellen Nielsen for help.

Received 30 July; accepted 10 September 1982.

- Kabat, E. A., Wu, T. T. & Bilofsky, H. in *Sequences of Immunoglobulin Chains. Tabulation and Analysis of Amino Acid Sequences or Precursors. V-Regions, C-Regions, J-Chain, and β_2 -Microglobulin* (Government Printing Office Publication, NIH-80-2008, 1979).
- Mage, R. in *Contemporary Topics in Molecular Immunology* Vol. 8 (eds Inman, F. R. & Mandy, W. J.) 89-112 (Plenum, New York, 1981).
- Kindt, T. J. *Adv. Immun.* **21**, 35-86 (1975).
- Mage, R. G. in *Progress in Immunology III*, 289-297 (Australian Academy of Science, Canberra, 1977).
- Margolies, M. N., Cannon, E. L., Kindt, T. J. & Fraser, B. J. *Immun.* **119**, 287-294 (1977).
- Siebenlist, U., Ravetch, J. V., Korsmeyer, S., Waldmann, T. & Leder, P. *Nature* **294**, 631-635 (1981).
- Wu, T. T. & Kabat, E. A. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5031-5032 (1982).
- Matthysens, G. & Rabbits, T. H. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6561-6565 (1980).
- Egel, R. *Nature* **290**, 191-192 (1981).
- Baltimore, D. *Cell* **24**, 592-594 (1981).
- Kabat, E. A., Wu, T. T. & Bilofsky, H. J. *exp. Med.* **149**, 1299-1313 (1979).
- Kabat, E. A., Wu, T. T. & Bilofsky, H. J. *exp. Med.* **152**, 72-84 (1980).
- Wu, T. T. & Kabat, E. A. *J. exp. Med.* **132**, 211-250 (1970).
- Bernstein, K. E. *et al. Molec. Immun.* (in the press).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 498-560 (1980).
- Ravetch, J. V., Siebenlist, U., Korsmeyer, S., Waldmann, T. & Leder, P. *Cell* **27**, 583-591 (1981).
- Tonnelle, C., Cazenave, P. A., Brezin, C. & Fougereau, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7088-7090 (1981).
- Sakano, H., Kurosawa, Y., Weigert, M. & Tonegawa, S. *Nature* **290**, 562-565 (1981).
- Kurosawa, J. & Tonegawa, S. *J. exp. Med.* **155**, 201-218 (1982).
- Knight, K. L., Malek, T. R. & Dray, S. *Nature* **253**, 216-217 (1975).
- Bodmer, W. F. *Transplant Proc.* **5**, 1471-1475 (1973).
- Strosberg, A. D. *Immunogenetics* **4**, 499-513 (1977).
- Roux, K. H. *Surv. immun. Res.* (in the press).

HMG-1	G K G D P K P R G K M S S Y A F F V Q T S R E E H K K H P D A S V N F S E x x x x x
HMG-2	G K G D P N K P R G K M S S Y A F F V Q T S R E E H K K H P D A S V N F S E x x x x x
HMG-1	D P N A P K R P P S A F F L F A S E Y R P K I K G E H P G L S I - - - - -
HMG-2	D P N A P K R P P S A F F L F A S E Y R P K I K A E H P G L S I - - - - -
	100 110 120
HMG-1	x x x x x x x x R W K T M S A K E K G K F E D M A K A D K A R Y E R E M K T Y I P P K G E T
HMG-2	x x x x x x x x R W K T M S A K E K S F E D M A K S D K A R Y D R E M K N Y V P P K G K
HMG-1	G D V A K L G E M W N N T A A D D K Q P Y E K K A A L K E K Y E K x A A Y R A K G K P D A
HMG-2	G D T A K L G E M W S Q Q S A K D K Q P Y E Q K A S K L K E L Y E K x A A Y R A K G K S E A
	130 140 150 160 170

Fig. 1 Internal homology in HMG-1,2. The amino acid sequences of HMG-1 and HMG-2 were treated as a two-member family and numbered as in ref. 5. The sequence at positions 40–53 and at position 165 is unknown (x). The single gap in the alignment is indicated by a series of dashes. The Sankoff algorithm was applied to the first two 100-residue portions of the family's sequence to produce the alignment shown. The 25 positions in the alignment at which the McLachlan score was from 7 to 10 (that is, positions where strong similarity exists between amino acid residues) are indicated by solid lines. The 15 positions at which weaker similarity exists (McLachlan scores of 5 or 6) are indicated by broken lines. Of the remaining 30 aligned positions, only 8 had McLachlan scores less than the average score of 4 obtained by pairwise comparison of all positions in the first homology region with all positions in the second homology region. There are apparently more residues in the stretch of unknown sequence (positions 39–54) in the first homology region than in the corresponding portion of the second homology region (positions 130–139). Thus, when the entire sequence of HMG-1,2 is determined, a gap will be necessary in the second homology region to optimize the entire alignment. In the meantime, we have arbitrarily placed positions 130–139. When allowed two gaps, the Sankoff algorithm introduces a five residue gap in the second homology region after position 164, but without a statistically significant increment⁶ in score. We therefore present the one gap alignment.

Table 1 Amino acid composition of fragment P3

	P3 (residues per 10,100 daltons)	HMG-1 (residues in 1–92)
Asp	7.9	5
Thr	2.4	4
Ser	6.1	9
Glu	8.7	12
Pro	4.8	5
Gly	6.2	6
Ala	7.8	7
Cys/2	1.9	1
Val	3.4	2
Met	4.2	4
Ile	0.5	1
Leu	0.6	0
Tyr	3.4	4
Phe	4.8	5
Trp	N.D.	1
His	2.3	2
Lys	17.5	19
Arg	5.8	5

The analysis of P3 was performed after 22 h of hydrolysis at 110 °C in 6 N HCl. No corrections have been made for partial destruction of Thr, Ser, Cys or Tyr. Trp was not determined. The amino acid composition of the N-terminal 92 residues of HMG-1 was calculated from the amino acid sequence of HMG-1 (ref. 5).

bility of obtaining a score by chance that is 8.3 or more standard deviations from the mean $[(1,079-604)/57 = 8.3]$ is 10^{-16} (obtained by interpolation of data compiled in ref. 13). This very low apparent probability emphasizes that the alignment in Fig. 1 is statistically highly significant.

Much of the sequence of the final portion (positions 177–259) of HMG-1,2 is not yet determined. However, that which has been sequenced is extraordinarily acidic. In HMG-1, over 50% of the positions sequenced are either Glu or Asp⁵, and a continuous stretch of 41 acidic residues has been reported¹⁴. Clearly, the final third of HMG-1,2 is not homologous in amino acid sequence with the two regions shown in Fig. 1.

The above analysis suggests a domain structure for HMG-1 and HMG-2 and, presumably, for the homologous¹⁵ HMG-E. We propose that all the high molecular weight HMG proteins consist of three domains. In HMG-1,2, the first domain, domain A, extends from position 1 to position 92. Domain B, which is homologous in sequence to domain A, corresponds to positions 98–176. Positions 93–97 (Lys-Lys-Lys-Phe-Lys in HMG-1) connect the first two domains. Domain C is the highly acidic C-terminal third of the proteins (positions 177–259). Domains A and B carry a net positive charge at neutral pH. In HMG-1, the number of Lys and Arg residues exceeds the number of Asp and Glu residues by 12 and 6 in domains A and B respectively. Domains A and B therefore seem well suited to be DNA-binding domains. Because they contain a normal complement of hydrophobic residues, these domains appear likely to occur in compact, globular conformations. Domain C, with its extreme content of acidic residues and paucity of hydrophobic residues (only six with marked hydrophobic character), presumably occurs as a coil, without organized folding. One would not expect it to bind to DNA and that expectation is supported experimentally¹⁶. Domain C seems to be designed, instead, to interact electrostatically with positively charged molecules such as histones. It is noteworthy that polyglutamic acid is capable of facilitating the assembly of core histones and DNA into nucleosomes¹⁷.

By applying the Chou and Fasman prediction rules¹⁸ to the sequences of domains A and B, we can predict their secondary structures. The predictions are strikingly similar for the two putative domains (Fig. 2). Each is predicted to have a substantial content of α -helix, in corresponding positions, and no β -sheet. The prediction is therefore consistent qualitatively with esti-

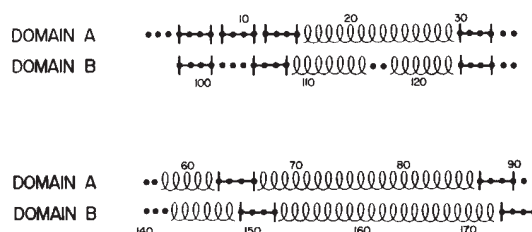


Fig. 2 Predicted secondary structure in domains A and B of HMG-1. The two portions of HMG-1 are aligned as in Fig. 1. Secondary structure, predicted by the rules of Chou and Fasman¹⁷, is shown only for the positions that are paired in Fig. 1. Residues predicted to occur in α -helices are shown as α . Nonhelical residues. Sets of four residues predicted to occur as β -turns (probability $p_i \geq 1.2 \times 10^{-4}$) are shown as solid lines. β -sheet potential exists in several short stretches in the HMG-1 sequence, but is not strong enough or does not extend long enough to over-rule α -helical potential in the same regions. The similarities in the predicted secondary structures of domains A and B extend, in the following ways, beyond those that are obvious from the diagram. (1) A third β -turn is predicted in the initial portion of domain B, but is not shown because it has a probability, $p_i = 1.1 \times 10^{-4}$, somewhat lower than the cut-off probability we used for predicting β -turns. In addition, that predicted turn overlaps at residue 101 with the turn predicted in domain A at residues 98–101. (2) The first predicted helical region in domain B is interrupted at positions 116 and 117. A corresponding weakness in the predicted helix occurs in domain A at residues 21–23: the helical potential of those residues is just above the threshold for helix termination. In these positions in each domain, there is β -turn potential, but with probabilities below our cut-off. (3) Residues 36–39 in domain A and residues 130–139 in domain B are not shown in the diagram because they are not aligned in Fig. 1; each region is, however, predicted to be helical. (4) A β -turn is predicted at positions 170–173 in domain B at a probability of 1.25×10^{-4} . The turn, which would correspond almost precisely with a turn predicted in domain A, is not shown because it overlaps with the turn predicted at positions 173–176, which has a higher probability. A similar case holds at positions 146–149 in domain B. Finally, note that positions 55–57 are predicted to be nonhelical because the residues there (Arg, Trp and Lys) all occur with high frequency in nonhelical regions adjacent to N-terminal portions of helices¹⁷. The final prediction at those positions could be affected by the identity of residues, now unknown, at positions 39–54 in the sequence of HMG-1.

mates of secondary structure in HMG-1 from circular dichroism measurements^{16,19}. The similarities in predicted structure extend to the positions of β -turns, even though the residues involved in corresponding predicted β -turns are not generally conserved in sequence between the two homologous regions.

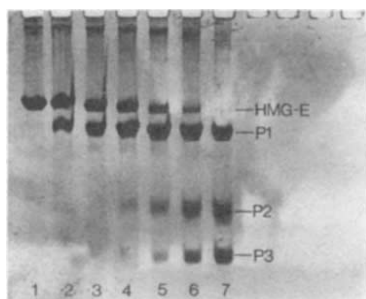


Fig. 3 Acid/urea polyacrylamide gel electrophoresis of the products of trypsin digestion of chicken erythrocyte HMG-E. HMG-E was purified by phosphocellulose chromatography²¹ of a 0.35 M NaCl extract of chromatin. The digestion was carried out at 0 °C in 0.1 M NaCl, 10 mM Tris-Cl, pH 7.5 with HMG-E at 25 µg ml⁻¹ and trypsin at 0.25 µg ml⁻¹. At the following times (min) aliquots were removed from the reaction mixture and samples prepared for electrophoresis as previously described⁴: 0 (track 1), 2 (track 2), 5 (track 3), 15 (track 4), 30 (track 5), 60 (track 6) and 120 (track 7). Material cleaved from HMG-E in forming P1 has not been detected on either acid/urea or SDS-polyacrylamide gel electrophoresis, perhaps because of failure to react with Coomassie blue or because of rapid diffusion from gels during staining or destaining.

Apart from X-ray crystallographic structure determination, the most direct experimental evidence for domain structure in proteins is the production, by limited proteolysis, of large, discrete fragments that are soluble in aqueous solvents. We have observed such fragments by treating individual high molecular weight HMG proteins with trypsin. Figure 3 shows an electrophoretic analysis of products of the trypsin cleavage of chicken erythrocyte HMG-E. Trypsin digestion mixtures have been subjected to chromatography on phosphocellulose and DNA-cellulose. P1, P2 and P3 are all water-soluble, DNA-binding proteins. We have purified P1 and P3 for analyses. P1 has a molecular weight of 19,000, as determined by sedimentation equilibrium in the analytical centrifuge. The molecular weight of P3 has been estimated to be 10,500 (by SDS-gel analyses) and 10,100 (by sedimentation equilibrium). P1 and P3 both have the same N-terminal amino acid sequence as the parent protein, HMG-E²⁰. (Ten residues were identified in HMG-E, in P1 and in P3 by automated Edman degradation.) P1 thus corresponds to domains A and B linked together, and P3 to domain A alone (Table 1). It has not yet proved possible to obtain P2 in sufficient purity or yield for analysis. Because P2 appears in comparable amounts to P3 and with the same time course as P3, and because P2 has a molecular weight of about 10,000 (by SDS-gel analysis), P2 probably corresponds to domain B.

The trypsin digestion results are thus consistent with our postulated three-domain structure for the high molecular weight HMG proteins. Studies of the structure and DNA-binding properties of the trypsin-generated fragments should provide insights into the role of the intact proteins in chromatin structure and function.

We thank Dr George Long for supplying a program for the Chou and Fasman prediction rules, and M. Daniel Land, David Manning, Lisa Wen and William Debold for assistance in some of the experiments. This work was supported by NIH grant R01-GM-13401 to D.C.T., by the Kansas Agricultural Experiment Station and by NIH grants R01-GM-29203, R01-CA-17782 and K04-CA-00425 to G.R.R. Publication 82-265-j, Kansas Agricultural Experiment Station.

Received 23 February; accepted 7 September 1982.

- Goodwin, G. H., Sanders, C. & Johns, E. W. *Eur. J. Biochem.* **38**, 14-19 (1973).
- Bidney, D. L. & Reeck, G. R. *Biochem. biophys. Res. Commun.* **82**, 1211-1218 (1978).
- Isackson, P. J., Fishback, J. L., Bidney, D. L. & Reeck, G. R. *J. Biol. Chem.* **254**, 5569-5572 (1979).
- Isackson, P. J. & Reeck, G. R. *Nucleic Acids Res.* **9**, 3779-3791 (1981).

- Walker, J. M., Gooderham, K., Hastings, J. R. B., Mayes, E. & Johns, W. E. *FEBS Lett.* **122**, 264-270 (1980).
- de Haën, C., Swanson, E. & Teller, D. C. *J. molec. Biol.* **106**, 639-661 (1976).
- Reeck, G. R., Swanson, E. & Teller, D. C. *J. molec. Biol.* **10**, 309-317 (1978).
- Sankoff, D. *Proc. natn. Acad. Sci. U.S.A.* **69**, 4-6 (1972).
- Sankoff, D. & Cedergren, R. J. *J. molec. Biol.* **77**, 159-164 (1973).
- McLachlan, A. D. *J. molec. Biol.* **61**, 409-424 (1971).
- de Haën, C., Neurath, H. & Teller, D. C. *J. molec. Biol.* **92**, 225-259 (1975).
- Birnbaum, Z. W. *Computers and Unconventional Test Statist. tech. Rep. 62* (Office of Naval Research, Contract N-onr-477(38), Seattle, 1973).
- Pearson, E. S. & Hartley, H. O. (eds) *Biometrika Tables for Statistics* Vol. 1, 3rd edn 117 (Cambridge University Press, 1966).
- Walker, J. M., Hastings, J. R. B. & Johns, E. W. *Nature* **271**, 281-282 (1978).
- Romani, M., Rodman, T. C., Vidali, G. & Bustin, M. *J. biol. Chem.* **254**, 2918-2922.
- Cary, P. D. *et al. Eur. J. Biochem.* **62**, 583-590 (1976).
- Stein, A., Whitlock, J. P. & Bina, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5000-5004 (1979).
- Chou, P. Y. & Fasman, G. D. *Adv. Enzym.* **47**, 45-148 (1978).
- Baker, C., Isenberg, I., Goodwin, G. H. & Johns, E. W. *Biochemistry* **15**, 1645-1649 (1976).
- Isackson, P. J. Thesis, Kansas St. Univ. (1981).
- Isackson, P. J., Debold, W. A. & Reeck, G. R. *FEBS Lett.* **119**, 337-342 (1980).

Electron microscopic localization of the replication origin of *Euglena gracilis* chloroplast DNA

P. Ravel-Chapuis, P. Heizmann & V. Nigon

Département de Biologie Générale, Université Claude Bernard Lyon-I, F-69622 Villeurbanne Cedex, France

Chloroplast DNA (ctDNA) generally occurs as circular molecules with molecular weights (MWs) in the range 70-130 × 10⁶ depending on the species^{1,2}. In *Euglena gracilis*, ctDNA (44 µm, 92 × 10⁶ MW³) replicates through Cairns-type intermediates⁴ having structural aspects suggesting bidirectional replication⁵. Pea and corn ctDNA were shown to contain two displacement loops (D-loops) located 7,100 base pairs (bp) apart⁶. The displacing strands of the two D-loops are located on opposite strands of the parental DNA; they expand towards each other and form a Cairns replicative intermediate when the two strands elongate past each other. Rolling circle intermediates⁷, apparently resulting from the continuation of Cairns rounds of replication, have also been observed⁸. The origins of replication were not located on physical maps for any of these studies. We present here the results of an electron microscopic (EM) study indicating that replication of ctDNA in *Euglena* is initiated near the 5' end of the supplementary 16S ribosomal RNA gene⁹.

Replicating ctDNA was extracted from *Euglena* cells synchronized by light-dark cycles (12 h/12 h)⁵ (Fig. 1). The highest yields of replicating molecules (up to 20% of the circular molecules) were obtained from cells collected in the middle of the light phase (compare with results of refs 5, 10). The replicated fraction of the various molecules represented from 1 to 80% of the total genome length.

When replicating ctDNA was digested with the restriction endonuclease *PvuII*, replication loops were observed on fragments 22.4 ± 0.3 µm long ($n = 31$ molecules) (Fig. 1a, b) corresponding to the fragment *Pvu* A (67 kilobase pairs, kbp; Fig. 2). After *XhoI* digestion replication loops were localized on fragments 16.2 ± 0.3 µm long ($n = 14$) (Fig. 1c) corresponding to the fragment *Xho* A (49 kbp; Fig. 2). These observations, taken together show that replication is initiated in the region common to *Pvu* A and *Xho* A fragments, that is, in the region 5' to the ribosomal cistrons (see physical map; Fig. 2). To ascertain this localization, R-loops were formed by hybridizing 23S ribosomal RNA (rRNA) with *PvuII* digests of replicating ctDNA. In agreement with the restriction map of Fig. 2, R-loops were observed on *Pvu* A fragments only. The region containing the three 23S cistrons is 4.7 ± 0.1 µm long ($n = 14$) while the adjacent segments spanning 3' and 5' of

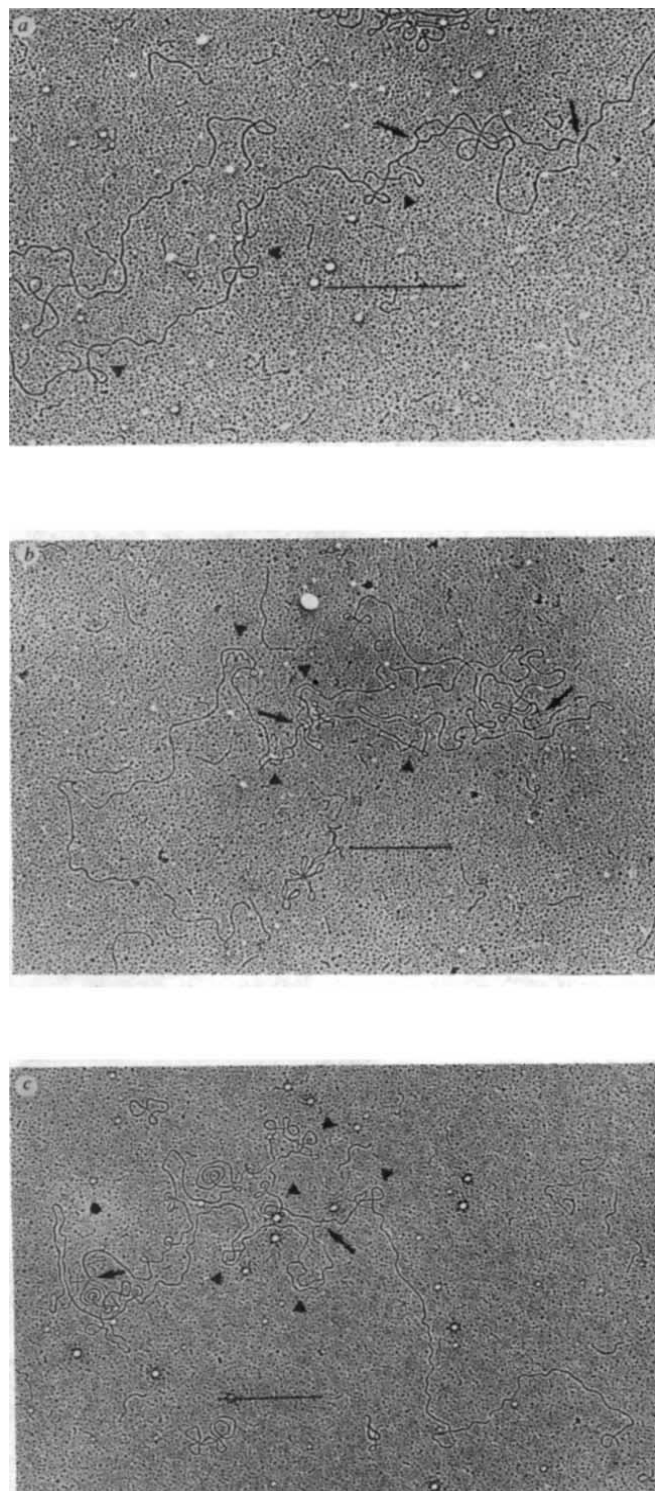


Fig. 3 *Pvu A* restriction fragments bearing simultaneously R-loops and a replication loop. ctDNA was prepared, digested and spread as described for Fig. 1. rRNA was phenol-extracted from SDS-lysed chloroplasts and 23S RNA was purified by 1.5% agarose gel electrophoresis, electroelution and alcohol precipitation. R-loops were formed according to Wellauer and Dawid²³ (incubation for 16 h at 45 °C). Arrows indicate R-loops. *a*, Replication concerns 5% of the total genome but not the ribosomal cistrons. A details of the ribosomal and replicated region is shown. *b*, Replication concerns 18% of the genome; the first 23S cistron has been replicated. *c*, 29% of the total genome and two 23S cistrons have been replicated. These molecules show branch migrations at one replication fork. Small circular molecules are pBR322 plasmid DNA. Scale bar, 1 μm.

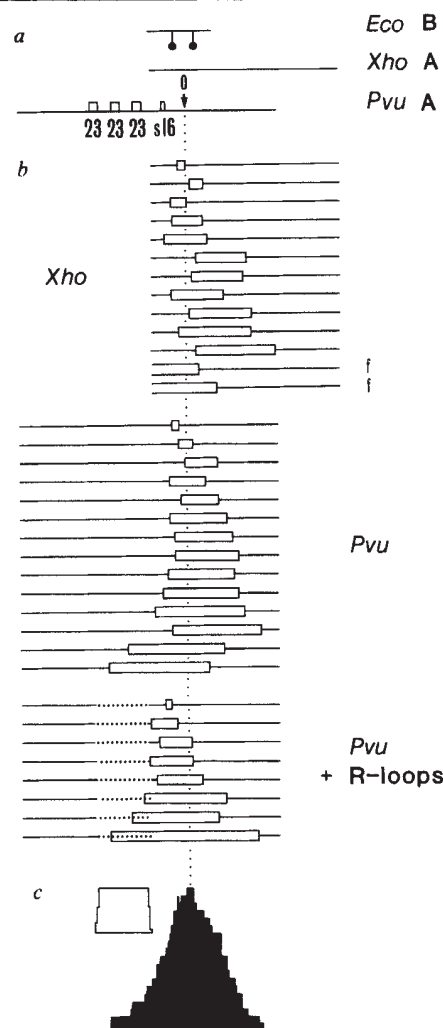


Fig. 4 Alignment of replication patterns with the physical restriction map. *a*, Relative positions of restriction fragments *Pvu A*, *Xho A* and *Eco B* from the physical map of Hallick¹² (Fig. 2). ● Represents the limits of the *Bgl Z* region as defined by Jenni *et al.*¹³. The positions of ribosomal 23S and supplementary 16S cistrons are indicated on the *Pvu A* fragment; 0 corresponds to the top of the histogram from *c*. *b*, Restriction fragments carrying replication loops. Restriction fragments were analysed by measuring the non-replicated portions and both branches of the replication loops of each individual restriction fragment. For all molecules observed, the two branches of the replication loops were identical in length ($\pm 5\%$); individual restriction fragments had total lengths within $\pm 7\%$ of the average fragment length. Only replication loops larger than 0.5 μm were taken into account to avoid possible confusion with denaturation loops, which are generally rare and smaller than 400–500 bp (~ 0.15 μm). Open boxes represent the replication bubbles; dotted lines represent the region covered by 23S R-loops. In each group molecules are arranged according to increasing extent of replication. *f*, Forked molecules. *c*, Histogram of cumulated *Pvu A* fragments from *b*. The open histogram indicates the position of the 23S ribosomal region observed on *Pvu* fragments.

being carried by each of the segments excised and repeated in spontaneous petites mutants²⁴. In an analogous way, ribosomal DNA amplification in *Tetrahymena* seems to proceed after excision of a rDNA segment from the micronucleus chromosomal DNA during conjugation, followed by extensive replication of extrachromosomal rDNA molecules due to the presence of an origin of replication located on the excised segments²⁵. Similarly, the tandem arrangement of the ribosomal cistrons in *Euglena*, together with their close proximity to the replication origin, could explain the relative amplification of these ribo-

somal cistrons described during mutagenesis²⁶ or suggested during the cell cycle^{27,28}.

This work was supported by CNRS grant ATP 8068. EM observations were performed at the Electron Microscopy Center of the Université Lyon-I.

Received 4 August; accepted 15 September 1982.

- Bedbrook, J. R. & Kolodner, R. A. *Rev. Pl. Physiol.* **30**, 593–620 (1979).
- Herrmann, R. G. & Possingham, J. V. in *Chloroplasts* (ed. Reinert, J.) 45–96 (Springer, 1980).
- Manning, J. E. & Richards, O. C. *Biochim. biophys. Acta* **259**, 285–296 (1972).
- Cairns, J. *Cold Spring Harb. Symp. quant. Biol.* **28**, 43–46 (1963).
- Richards, O. C. & Manning, J. E. in *Les Cycles Cellulaires* 213–221 (Colloques Internationaux du CNRS, Editions du CNRS, 1975).
- Kolodner, R. & Tewari, K. K. *J. biol. Chem.* **250**, 8840–8847 (1975).
- Gilbert, W. & Dressler, D. *Cold Spring Harb. Symp. quant. Biol.* **33**, 473–484 (1968).
- Kolodner, R. & Tewari, K. K. *Nature* **256**, 708–711 (1975).
- Jenni, B. & Stutz, E. *FEBS Lett.* **102**, 95–99 (1979).
- Brandt, P. *Planta* **124**, 105–107 (1975).
- Clayton, D. A. *Cell* **28**, 693–705 (1982).
- Hallick, R. B. in *The Biology of Euglena* Vol. 4 (ed. Buetow, D. E.) (Academic, New York, in the press).
- Jenni, B., Fasnacht, M. & Stutz, E. *FEBS Lett.* **125**, 175–179 (1981).
- Koller, B. & Delius, H. *FEBS Lett.* **139**, 86–92 (1982).
- Kroon, A. M. *et al. Biochim. biophys. Acta* **478**, 128–145 (1977).
- Bibb, M. J., Van Etten, R. A., Wright, C. T., Walberg, M. W. & Clayton, D. A. *Cell* **26**, 167–180 (1981).
- Anderson, S. *et al. Nature* **290**, 457–465 (1981).
- Ramirez, J. L. & Dawid, I. B. *J. molec. Biol.* **119**, 133–146 (1978).
- Wolstenholme, D. R., Goddard, J. M. & Fauron, C. M. R. in *Extrachromosomal DNA* (eds Cummings, D. J., Borst, P., Dawid, I. B., Weissman, S. M. & Fox, C. F.) 409–425 (Academic, New York, 1979).
- Hirota, Y. *et al. Prog. Nucleic Acid Res. molec. Biol.* **26**, 33–48 (1981).
- Moore, D. D., Denniston, K. J. & Blattner, F. R. *Gene* **14**, 91–101 (1981).
- Subramanian, K. N., Dhar, R. & Weissman, S. M. *J. biol. Chem.* **252**, 355–367 (1977).
- Hartley, J. L. & Donelson, J. E. *Nature* **286**, 860–865 (1980).
- de Zamaroczy, M. *et al. Nature* **292**, 75–78 (1981).
- Pan, W.-C. & Blackburn, E. H. *Cell* **23**, 459–466 (1981).
- Heizmann, P., Hussein, Y., Nicolas, P. & Nigon, V. *Curr. Genet.* **5**, 9–15 (1982).
- Gibson, W. H. & Hershberger, C. L. *Archs Biochem. Biophys.* **168**, 8–14 (1975).
- Manning, J. E. & Richards, O. C. *Biochemistry* **11**, 2036–2043 (1972).
- Freysinet, G., Heizmann, P., Verdier, G., Trabuchet, G. & Nigon, V. *Physiol. Vég.* **10**, 421–442 (1972).
- Davis, R. W., Simon, M. & Davidson, N. *Meth. Enzym.* **21**, 413–428 (1971).
- Younghusband, H. B. & Inman, R. B. *A. Rev. Biochem.* **43**, 605–618 (1974).
- Wellauer, P. K. & Dawid, I. B. *Cell* **10**, 193–212 (1977).

Impaired nuclear transport of a human variant tRNA_i^{Met}

Michael Zasloff^{*,†}, Martin Rosenberg[†]
& Tomas Santos^{*,†}

^{*} Genetics and Biochemistry Branch, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, and [†] Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, USA

Recently, a human variant tRNA_i^{Met} (initiator tRNA^{Met}) gene has been cloned from a recombinant library of fetal liver DNA¹. This gene contains a G to T transversion within the highly conserved TCGA sequence in loop 4 ('Tψ loop'), a sequence position occupied exclusively by a purine (usually G) in almost 200 prokaryotic and eukaryotic tRNAs². Recent studies with the cloned gene, both *in vitro*³ and when expressed in an intact cell on a simian virus 40 viral vector⁴, have shown that one functional consequence of this base substitution is a reduction in the rate of processing of the primary transcript of the gene. This reaction involves sequential excision of 5'- and 3'-terminal sequences³. Here we show that following microinjection of the variant tRNA_i^{Met} gene into the germinal vesicle of the intact *Xenopus laevis* oocyte, the primary transcript is slowly but accurately processed to a mature variant tRNA_i^{Met} species which remains trapped within the oocyte nucleus, its escape apparently being prevented by the nuclear membrane. This is the first evidence that the transport of a tRNA molecule can be blocked by a point mutation. The results suggest that a selective tRNA nuclear transport mechanism exists in the eukaryotic cell and identify a critical role for the highly conserved Tψ loop in this process.

[†] Present addresses: Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205, USA (M.Z.) and Department of Microbiology, University of Salamanca, Spain (T.S.).

The *X. laevis* oocyte has been shown to accurately transcribe and process several homologous and heterologous tRNA genes^{5–9} and provides a system for studying the intracellular distribution of intermediates in tRNA biosynthesis. After microinjection of either a cloned tRNA gene or a purified transcript, the oocyte can be manually dissected and the RNA species within the nucleus and cytoplasm analysed separately^{8,9}. The intracellular distribution of tRNA intermediates is preserved during manual dissection, in contrast to the 'leakiness' of somatic nuclei with respect to these species¹⁰, when isolated by standard aqueous fractionation.

The DNA sequences of the two human tRNA_i^{Met} genes used in this study are co-linear with that of mature tRNA_i^{Met} and neither contains an intervening sequence¹; gene 1 is a variant differing from the common vertebrate tRNA_i^{Met} sequence by a G to T transversion at position 56 in the DNA sequence (corresponding to position 57 in the standard tRNA numbering scheme²), and gene 2 contains the common vertebrate sequence. The 5' and 3' flanking sequences of the two genes share only limited homology, with the corresponding primary transcripts of each gene differing in both the sequence and length of the 5' leader and 3' trailer³. Plasmids pH1E (gene 1) and pH2D (gene 2), described in detail elsewhere¹, each contain a human chromosomal fragment bearing a single tRNA gene.

The two plasmids were microinjected separately into the germinal vesicle of stage 6 *X. laevis* oocytes. Five hours after injection of the gene plus [α-³²P]GTP, RNA was extracted and

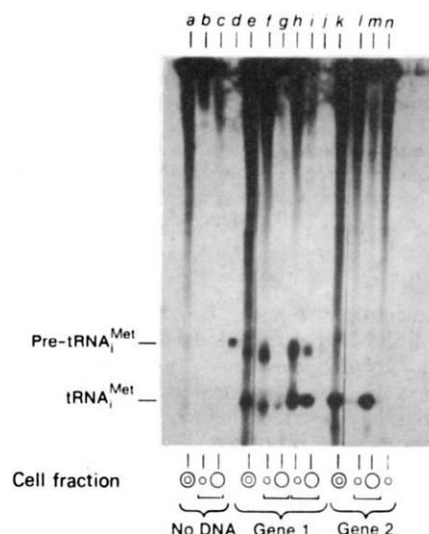


Fig. 1 Intracellular distribution of ³²P-labelled RNA species in *X. laevis* oocytes after microinjection of human tRNA_i^{Met} genes. Ovaries were collected from gravid *X. laevis* females and microinjected as described elsewhere⁵; ~20 nl of solution containing 10 ng of plasmid were injected per nucleus. Plasmids pH1E (gene 1) and pH2D (gene 2) have been described in detail elsewhere¹. Plasmids were isolated by acid-phenol extraction¹⁸ followed by gel filtration over Sepharose 4B. After injection of the plasmid, 50 nl of [α-³²P]GTP (10 mCi ml⁻¹; SA 760 Ci mmol⁻¹, NEN) were injected in a separate step into either the vegetal (white) pole or the equator. Oocytes were maintained at 21°C for 5 h. RNA was extracted from either the whole oocyte or the separated germinal vesicle and cytoplasm, manually dissected as described by Melton *et al.*⁹. RNA was electrophoresed on a 10% acrylamide gel containing 7 M urea and autoradiographed for 12 h as described³. No exogenous DNA injected: a, whole oocyte; b, germinal vesicle; c, cytoplasm. pH1E (gene 1) injected: d, whole oocyte; e, germinal vesicle; f, h, cytoplasmic fractions. pH2D (gene 2) injected: j, whole oocyte; k, germinal vesicle; l, n, cytoplasmic fraction associated with the vesicle in lane n is not presented). Lanes d and j, ³²P-labelled primary transcripts of gene 1 and gene 2, respectively, synthesized *in vitro*³ (see Fig. 3 legend), were included as size markers.

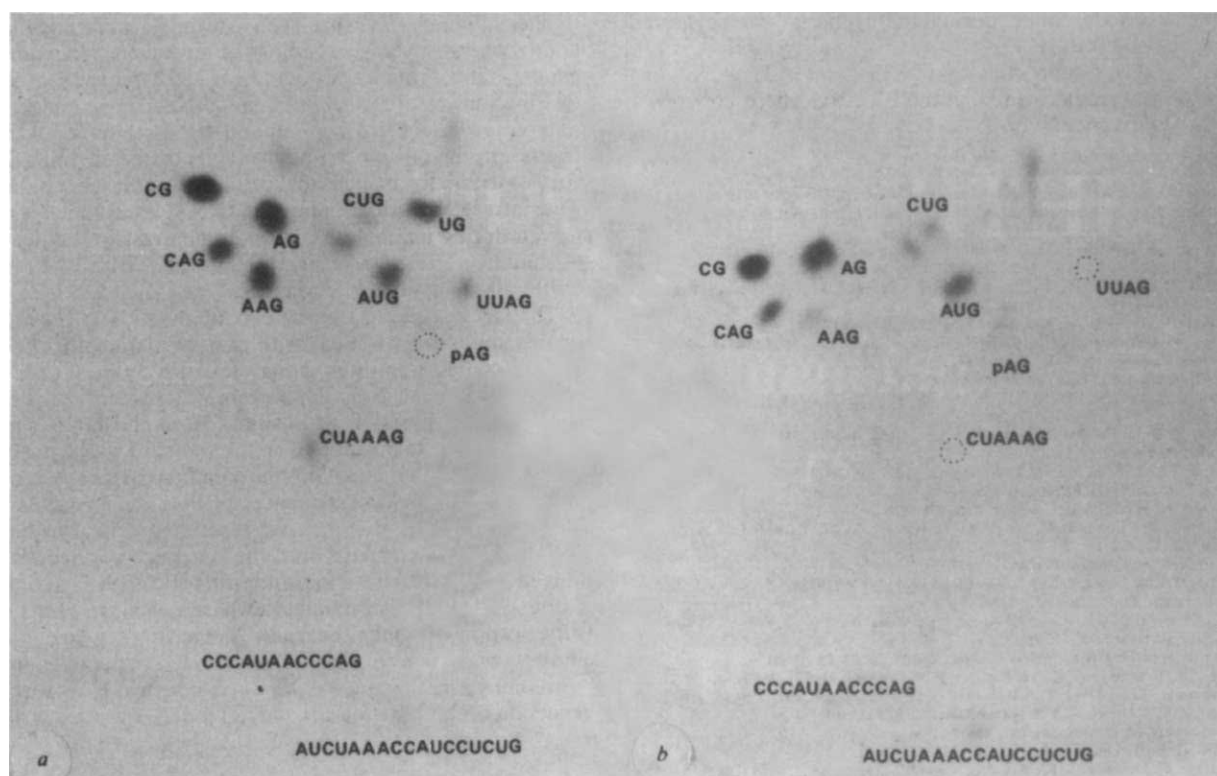


Fig. 2 RNase T₁ oligonucleotide fingerprint of gene 1 pre-tRNA_i^{Met} and intranuclear tRNA_i^{Met}. RNA species were extracted from the gel shown in Fig. 1*f* and fingerprinted by classical techniques as described elsewhere³. Expected positions of certain oligonucleotides not observed in each fingerprint are indicated by dotted circles. *a*, Pre-tRNA_i^{Met} transcribed from gene 1. The long oligonucleotide characteristic of the variant, AUCUAAACCAUCCUCUG, results from the elimination of an AUCG in loop 4 due to base substitution of G for U. The oligonucleotide CUAAG can also be seen, bridging the 3' end of the mature sequence and 3' trailer. UUAG arises from position -2 in the DNA sequence, bridging the two nucleotides of the 5' leader and two nucleotides from the start of the mature sequence. *b*, Intranuclear tRNA_i^{Met}. The characteristic long oligonucleotide is present, demonstrating the presence of the mutation within the loop 4 region. The 3' bridging sequence, CUAAG, is absent and the mature 5' end, pAG, is present.

analysed by electrophoresis in 10% acrylamide gels in 7 M urea. Figure 1 shows that injection of gene 2 resulted in the synthesis of an RNA species having the mobility expected for tRNA_i^{Met} (lane *k*); the structure was confirmed by RNA sequence analysis as described below. On injection of gene 1, two major species were produced (Fig. 1*e*); the slower is the precursor of the variant tRNA while the faster, major species, is mature variant tRNA_i^{Met}.

To determine the intracellular distribution of the transcripts of the normal and mutant tRNA_i^{Met} genes, the gene plus [α -³²P]GTP were injected into the germinal vesicle of an intact oocyte and kept at 21 °C for 5 h; the oocyte was then manually dissected and RNA species extracted from the separated nucleus and cytoplasm. The tRNA_i^{Met} species synthesized from the normal gene was found principally in the cytoplasmic compartment (Fig. 1*m*) with about 1% of the total newly synthesized tRNA_i^{Met} remaining in the germinal vesicle (Fig. 1*l, n*). As the volume of the germinal vesicle is about 10% that of the oocyte¹¹, the intranuclear concentration is lower than would be expected from free diffusion of the species between nucleus and cytoplasm and suggests that the tRNA_i^{Met} partitions preferentially in the cytoplasm.

The intracellular distribution of the RNA species transcribed from the mutant gene differed strikingly from that observed for normal tRNA_i^{Met}. As seen in the representative pair of oocyte dissections, the germinal vesicle contained, in addition to the precursor species, between 80% (Fig. 1, lanes *f, g*) and 60% (Fig. 1*h, i*) of the newly synthesized variant tRNA. Because tearing of the germinal vesicle membrane leads to leakage of the intranuclear tRNA species, as we show below, we cannot exclude the possibility that the cytoplasmic component was solely an artefact of isolation. Note, however, that

the relative proportion of the tRNA species present in each compartment did not change significantly with time up to 18 h.

The putative variant tRNA precursor, and the nuclear and cytoplasmic tRNA species were isolated and analysed by classical two-dimensional T₁ RNase oligonucleotide fingerprinting¹². The fingerprints generated were compared with previous analyses of the *in vitro* transcripts of this gene³. The fingerprint of the slowly migrating species (Fig. 2*a*) is characteristic of the precursor, while that of the intranuclear tRNA (Fig. 2*b*) is characteristic of the mature variant tRNA_i^{Met}. Fingerprint analysis of the tRNA synthesized in the presence of [α -³²P]CTP revealed an oligonucleotide with location and composition consistent with the mature 3'-terminal oligonucleotide CUACCA^{OH}, demonstrating terminal CCA addition on the accurately processed terminus (data not shown). A fingerprint of the variant tRNA recovered from the cytoplasm appeared identical to that of the intranuclear species (data not shown).

To determine whether the processing and transport behaviour of the mutant tRNA could be demonstrated with purified RNA species, ³²P-labelled primary transcripts of each gene synthesized *in vitro*³ were injected separately into individual oocytes. After 5 h incubation, the RNA was extracted from either the whole oocyte or from the germinal vesicle and cytoplasm after manual dissection. Injection of the purified primary transcripts resulted in a similar appearance and intracellular distribution of species observed during active transcription in the oocyte. Thus, the primary gene 2 transcript was fully converted to tRNA_i^{Met} (Fig. 3*k*) 5 h after nuclear injection. The processing nucleases appear to be intranuclear, because injection of the same quantity of precursor into the cytoplasm resulted in less efficient conversion (Fig. 3*l*). Analysis of the distribution of the tRNA_i^{Met} species revealed that virtually

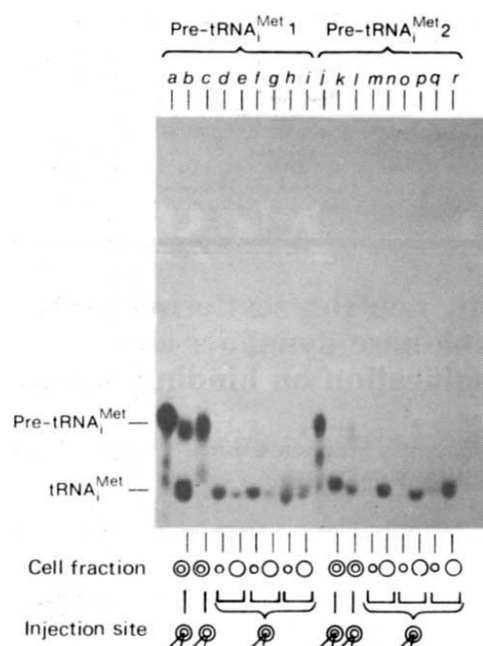


Fig. 3 Intracellular processing and distribution of ^{32}P -labelled tRNA_{Met} primary transcripts after microinjection of the purified RNA into *X. laevis* oocytes. The primary transcripts of gene 1 and gene 2 were synthesized *in vitro* using a partially purified KB cell postnuclear fraction as described previously³, and have been shown to be identical in sequence to the species synthesized in the *X. laevis* oocyte extract³. The gene 1 primary transcript, 90 nucleotides long, contains a 3-base 5' leader and a 13-base 3' trailer, while the 89-base gene 2 transcript contains an 8-base leader and trailer³. The RNA species were purified from the transcription reactions by phenol extraction, concentrated by ethanol precipitation, and then resuspended in a small volume of 88 mM NaCl, 20 mM Tris-HCl pH 8.0. RNA solutions were injected into oocyte nuclei, as described in Fig. 1 legend, or into the cytoplasmic pole. Following incubation at 21 °C for 5 h, RNA was extracted from either whole oocytes or nuclear and cytoplasmic fractions, obtained as described in Fig. 1 legend. *a*, Primary transcript of gene 1 synthesized *in vitro*. *b*, Whole oocyte, following injection into the germinal vesicle of the amount of ^{32}P -RNA shown in *a*. *c*, Whole oocyte, following injection into the cytoplasm. *d*, *f*, *h*, Germinal vesicles; *e*, *g*, *i*, corresponding cytoplasmic fractions following germinal vesicle injections. Lanes *j*–*r* show corresponding data obtained after injection of the primary transcript of gene 2.

no tRNA_{Met} remained within the nucleus; it was localized within the cytoplasm, as shown in three sets of single oocyte dissections (Fig. 3*m/n*, *o/p* and *q/r*).

The primary transcript of gene 1 was processed to a tRNA species more slowly than that of gene 2, and the variant tRNA_{Met} was inefficiently transported from the oocyte nucleus. About 50% of the primary transcript of gene 1 was processed to a tRNA by 5 h after microinjection into the germinal vesicle (Fig. 3*b*). We estimate that the rate of processing of this transcript is about 5% of that observed for the gene 2 species, since 50% conversion of the gene 2 transcript occurred within about 15 min after microinjection (data not shown). As demonstrated in three individual oocyte dissections, between 90% (Fig. 3*d/e* and *f/g*) and 60% (Fig. 3*h/i*) of the processed mutant tRNA_{Met} remained within the germinal vesicle 5 h after intranuclear injection of the gene 1 transcript. The gene 1 primary transcript was not significantly processed after injection into the cytoplasm of the oocyte (Fig. 3*c*), which demonstrates that the primary transcript of the variant gene cannot efficiently enter the nucleus from the cytoplasmic compartment during this period.

To determine the mechanism by which the tRNA_{Met} remains sequestered within the nucleus of the oocyte, we asked whether this mutant tRNA was limited from free diffusion by the nuclear

membrane, by physical attachment to a cellular component such as the membrane itself or the intranuclear matrix, or due to its association with a large soluble macromolecule which might itself be limited from leaving the germinal vesicle. Five hours after injection of gene 1 and [α - ^{32}P]GTP, the germinal vesicles were manually isolated and placed in a small volume of J buffer¹³ (70 mM NH_4Cl , 7 mM MgCl_2 , 0.1 mM EDTA, 2.5 mM dithiothreitol, 10 mM HEPES pH 7.4, 10% glycerol) or the same buffer containing 1% Triton X-100. [In the presence of this non-ionic detergent the nuclear membrane is disrupted without apparent effect on the basic overall structural integrity of the intranuclear matrix, and the surrounding pore lamina system or 'envelope'¹⁴. Figure 4 (inset) shows a low power magnification of a germinal vesicle suspended for 30 min at 21 °C in J buffer containing Triton X-100. No appreciable difference in gross morphology was observed between the germinal vesicle thus treated and one suspended in J buffer alone for this period of time.] After incubation of the isolated germinal vesicle in J buffer, the labelled RNA species remained within the nucleus (Fig. 4*c*) with little RNA found in the surrounding buffer (lane *d*). In contrast, treatment of the germinal vesicle with the solution containing Triton X-100 resulted in the almost complete release of the RNA species from the nucleus (Fig. 4*e*) to the surrounding solution (lane *f*).

Thus, treatment of the germinal vesicle in conditions which lead to dissolution of the nuclear membrane without gross physical disruption of the nucleus, results in leakage of the

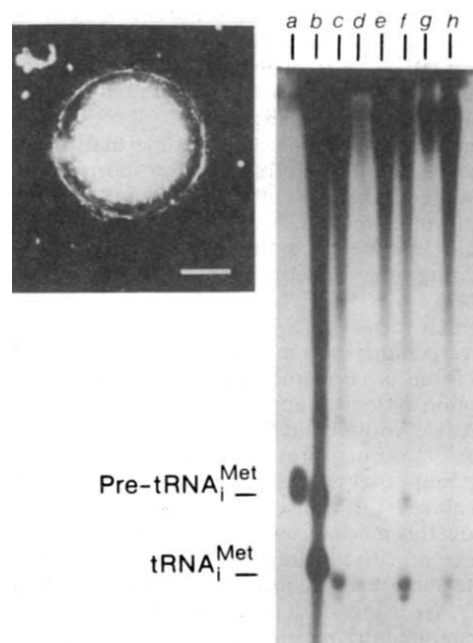


Fig. 4 Intranuclear variant tRNA_{Met} is trapped in the germinal vesicle by the nuclear membrane. ^{32}P -RNA was synthesized in oocytes after injection of pHIE (gene 1) as described in Fig. 1 legend. *a*, Gene 1 primary transcript synthesized *in vitro*. *b*, RNA extracted from whole oocyte. Germinal vesicles were isolated as described in Fig. 1 legend and suspended in 300 μl of either J buffer¹³ or J buffer containing 1% Triton X-100, and maintained at 21 °C for 30 min, at which time the RNA species present in both nucleus and surrounding buffer were analysed. Germinal vesicle suspended in J buffer alone: *c*, germinal vesicle; *d*, surrounding fluid. Germinal vesicle suspended in J buffer plus Triton X-100: *e*, germinal vesicle; *f*, surrounding fluid. One germinal vesicle was placed in J buffer and the nuclear membrane manually separated from the nucleoplasm: *g*, RNA associated with nuclear membrane; *h*, intranuclear contents. Inset, *X. laevis* germinal vesicle after incubation at 21 °C for 30 min in J buffer containing 1% Triton X-100. No change in gross morphology of the germinal vesicle was observed after detergent treatment. Note the nuclear envelope, nucleoplasmic gel, and numerous amplified nucleoli. (Nomarski phase optics; scale bar, 0.1 mm.)

variant tRNA_i^{Met}. To determine whether the RNA species is released as a result of the membrane acting as a diffusion barrier or as a result of previous attachment of the tRNA species to the membrane, a germinal vesicle, previously injected with gene 1 and [α -³²P]GTP, was placed in J buffer (not containing Triton X-100) and manually stripped of its enclosing nuclear envelope as described elsewhere¹³. When the contents of the nucleus were separated from the membrane, the variant tRNA_i^{Met} partitioned with the nucleoplasmic contents (Fig. 4h) rather than the membrane (lane g).

Velocity sedimentation analysis of the intranuclear contents of the manually stripped germinal vesicle revealed that the intranuclear tRNA_i^{Met} species has a mobility of ~4S (data not shown). This experiment demonstrates that the species is not associated within the nucleus in a large macromolecular complex (such as the 42S particle¹⁵) and, even in the absence of detergent treatment, is not bound tightly to such components as the nuclear gel, the intranuclear scaffold, or chromatin (nuclear and nucleolar).

We have demonstrated here that a human variant tRNA_i^{Met} containing a base substitution within the highly conserved loop 4 region is transported inefficiently from the oocyte nucleus. Although only tRNA species having mature 5' and 3' termini can leave the nucleus, as noted here and in an earlier study⁹, our data reveal that accurate nucleolytic processing of a tRNA molecule will not guarantee nuclear escape. In addition, the trapping of the variant tRNA_i^{Met} within the oocyte nucleus demonstrates that the nuclear membrane can restrict the diffusion of a mature tRNA species and provides evidence for the existence of a tRNA transport mechanism. The behaviour of the variant tRNA_i^{Met} strongly suggests that the highly conserved UCGA sequence within the loop 4 region of eukaryotic tRNA has a role in the overall transport of a tRNA molecule across the nuclear membrane. Our finding that the variant is processed slowly in addition to being transported inefficiently suggests that the mechanisms of processing and transport might be functionally inter-related. Alternatively, both processing and transport may operate by entirely independent mechanisms, each recognizing common sequences (or structure) in the tRNA molecule.

Is this human variant tRNA_i^{Met} a 'pseudogene' in the sense of an inactive member of a multigene family, or might it have a function? From *in vitro* studies of several RNA polymerase III transcription systems it appears that feedback inhibition by either tRNA (U. Affolter and M. Z., unpublished observations) or 5S rRNA^{16,17} occurs, presumably by the binding of RNA with a component that is present in rate-limiting amounts and which is required for transcription initiation. It is unclear, however, how this mechanism might operate in the intact cell, as tRNA once processed is rapidly excluded from the nucleus, with no detectable back transport of mature tRNA from the cytoplasm¹¹. We suggest that this human variant tRNA_i^{Met} (either as a mature intranuclear species, or as a slowly processed precursor) might provide the intranuclear transcription apparatus with a means of sensing the output of the multigene tRNA_i^{Met} family, perhaps by the mechanism suggested by the *in vitro* studies described.

We thank Joanne Nickol and William Bonner for technical advice during the initial phases of our oocyte studies, and Mrs Mary Keffer for help in preparation of the manuscript.

Received 21 June; accepted 16 September 1982.

1. Santos, T. & Zasloff, M. *Cell* **23**, 699-709 (1981).
2. Sprinzl, M. & Gauss, D. M. *Nucleic Acids Res.* **10**, r1-r55 (1982).
3. Zasloff, M., Santos, T., Romeo, P. & Rosenberg, M. *J. biol. Chem.* **257**, 7857-7863 (1982).
4. Zasloff, M., Santos, T. & Hamer, D. H. *Nature* **295**, 533-535 (1982).
5. Kressmann, A., Clarkson, S. G., Telford, J. L. & Birnstiel, M. L. *Cold Spring Harb. Symp. quant. Biol.* **42**, 1077-1082 (1977).
6. DeRobertis, E. M. & Olson, M. V. *Nature* **278**, 137-143 (1979).
7. Cortese, R., Melton, D., Tranquilla, T. & Smith, J. D. *Nucleic Acids Res.* **5**, 4593-4611 (1978).
8. Melton, D. & Cortese, R. *Cell* **18**, 1165-1172 (1979).

9. Melton, D., DeRobertis, E. M. & Cortese, R. *Nature* **284**, 143-148 (1980).
10. Lonn, U. *J. molec. Biol.* **112**, 661-666 (1977).
11. DeRobertis, E. M., Lienhard, S. & Parisot, R. *Nature* **295**, 572-577 (1982).
12. Barrel, B. G. in *Procedures in Nucleic Acid Research* Vol. 2 (eds Cantoni, G. L. & Davis, D. R.) 751-799 (Harper & Row, New York, 1971).
13. DeRobertis, E. M., Black, P. & Nishikura, K. *Cell* **23**, 89-93 (1981).
14. Kirschner, R. H., Rusli, M. & Martin, T. E. *J. Cell Biol.* **72**, 118-132 (1977).
15. Picard, B. & Wegnez, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 241-245 (1979).
16. Pelham, H. R. B. & Brown, D. D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4170-4174 (1980).
17. Gruissen, W. & Seifart, K. H. *J. biol. Chem.* **257**, 1468-1472 (1982).
18. Zasloff, M., Ginder, G. & Felsenfeld, G. *Nucleic Acids Res.* **5**, 1139-1151 (1978).

Inelastic neutron scattering analysis of hexokinase dynamics and its modification on binding of glucose

Bernard Jacrot*, Stephen Cusack*, Albert J. Dianoux† & Donald M. Engelman‡

* European Molecular Biology Laboratory, Grenoble Outstation, c/o ILL, 156X, 38042 Grenoble Cedex, France

† Institut Laue-Langevin, 156X, 38042 Grenoble Cedex, France

‡ Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, USA

The dynamical behaviour of proteins and its significance to protein function has become the subject of considerable experimental and theoretical investigation^{1,2}. The existence of atomic motion about mean positions has been well established by the temperature dependence of X-ray diffraction³ and it has long been recognized that considerable internal mobility is required in proteins to account for hydrogen exchange measurements⁴. However, only limited experimental information, mainly obtained by optical methods⁵, is available on the frequencies associated with these fluctuations. Inelastic neutron scattering is a technique capable of yielding dynamical information about a system over a wide frequency range^{6,7}. Recent advances in neutron spectrometers⁸ and in the theoretical understanding of the dynamics of biological macromolecules have now made it both feasible and desirable to exploit this technique in the field of biophysics. To demonstrate the potential of the method, we report here data on the frequency distribution in solution of the enzyme hexokinase and its modification brought about on binding of its substrate glucose. The results lend support to the view that ligation induces a stiffening of the enzyme structure.

Inelastic neutron scattering arises when incident neutrons gain or lose energy as a result of interaction with the internal modes of motion of a sample. Techniques to measure these energy changes are routinely used to study the dynamics of solids and liquids⁹. In the case of organic molecules the signal is dominated by the incoherent scattering by the hydrogen nuclei and can be related to the frequency spectrum of the sample⁷. However, the application of this technique to proteins in solution is hampered by the necessarily low protein concentration (at best a few per cent) which means that all measured spectra are dominated by the scattering by the buffer. Use of buffers made from D₂O minimizes this background, but despite this, several previous attempts to measure the inelastic spectra of proteins in solution have suffered from insufficient statistical accuracy. This has been overcome by the time focusing time-of-flight spectrometer IN6⁸ (see Fig. 1 legend) which has recently become available at the Institut Laue-Langevin, and enables measurements to be made with good counting statistics. Inelastic neutron scattering data on polycrystalline lysozyme have been reported previously⁹.

We have chosen yeast hexokinase (molecular weight 50,000) for this investigation for the following reasons. Its structure is known from X-ray crystallography¹⁰ and consists of two large domains with a deep cleft between them. A crystallographic study of an isozyme of hexokinase complexed with glucose has shown that in the complex the two domains have rotated so as

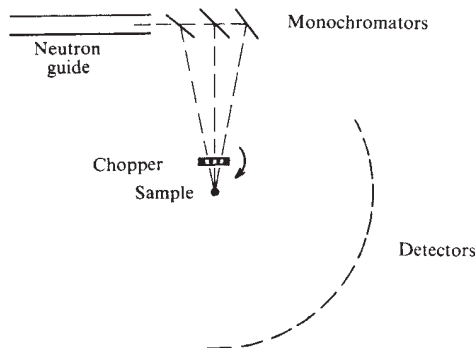


Fig. 1 Schematic diagram of time-focusing spectrometer IN6 viewed from above. Neutrons arriving through a guide tube ($3 \times 20 \text{ cm}^2$) are monochromatized by a series of three pyrolytic graphite crystals with slightly different orientations. Each crystal reflects a different wavelength but all beams are focused on the sample. Pulses of neutrons are produced by a chopper which rotates about a vertical axis and is placed 40 cm before the sample. The chopper is designed so that the wavelength differences between the sub-beams are compensated by different times of chopping. In this way all elastically scattered neutrons arrive at the detector at the same time (time focusing). The instrument thus combines a high flux with a reasonable resolution ($\sim 0.15 \text{ meV}$ or 1.2 cm^{-1} at the elastic peak). In the direction perpendicular to the diagram the neutron guide tube is 20 cm in height and a geometrical focusing of the beam in that direction is achieved by curved composite monochromators. The inelastically scattered spectrum is analysed by measuring the time of flight from the sample to a bank of detectors 247 cm away. The detectors were grouped to give 24 time-of-flight spectra corresponding to scattering angles between 10° and 115° . Each time-of-flight spectrum had 512 channels. Incident neutrons of wavelength $4.1 \text{ \AA}$ (4.87 meV) were used.

to trap the glucose in the cleft^{11,12}. That this conformational change on binding of glucose actually occurs in solution has been confirmed by measuring the corresponding change in the radius of gyration using small-angle X-ray scattering¹³. The interest in making inelastic neutron scattering measurements on this system follows from the speculation that the binding of glucose in the cleft and resultant conformational change may modify the vibrational spectrum of hexokinase. In particular, relative motion of the two domains may be hindered on ligation. Furthermore, it has been argued that a change in the frequency spectrum of an enzyme on ligation may be significant in the thermodynamics of enzymatic reactions¹⁴.

The experimental strategy was to collect data successively from the buffer, the hexokinase solution and the hexokinase saturated with glucose, all at 15°C and in the same quartz cell. Figure 2 shows corrected and scaled spectra obtained by summing counts in the range of scattering angle between 84° and 110° . The spectra of the hexokinase solution and the buffer appear similar but the statistical accuracy is sufficiently high to show that the enzyme spectrum is significantly different in form from the unsubtracted spectrum. The signal from the protein is about 15% of the total. In subtracting the buffer a factor was introduced to take into account the lower buffer content of the protein solution. We verified that variation of this scaling factor by $\pm 5\%$ (a wider margin of error than the true error) did not significantly affect the form of the protein spectrum (in particular the peak around 50 cm^{-1}). Thus, we believe that curves *c* and *d* in Fig. 2 are an accurate representation of the inelastic neutron spectra of hexokinase and hexokinase bound with glucose. Spectra were simultaneously measured at several scattering angles between 10° and 115° , and the angular dependence of the spectra can be summarized as follows. The intensity of the elastic peak of both the ligated and unligated spectra decrease roughly as $\exp(-0.89Q^2)$. The two main features of the inelastic spectrum—the peaks around 50 and 300 cm^{-1} —behave differently. The former increases in intensity with angle while the latter maintains the same intensity and is hardly distinguishable as a distinct peak at a scattering angle of 97° . This different behaviour with angle of high and low frequency

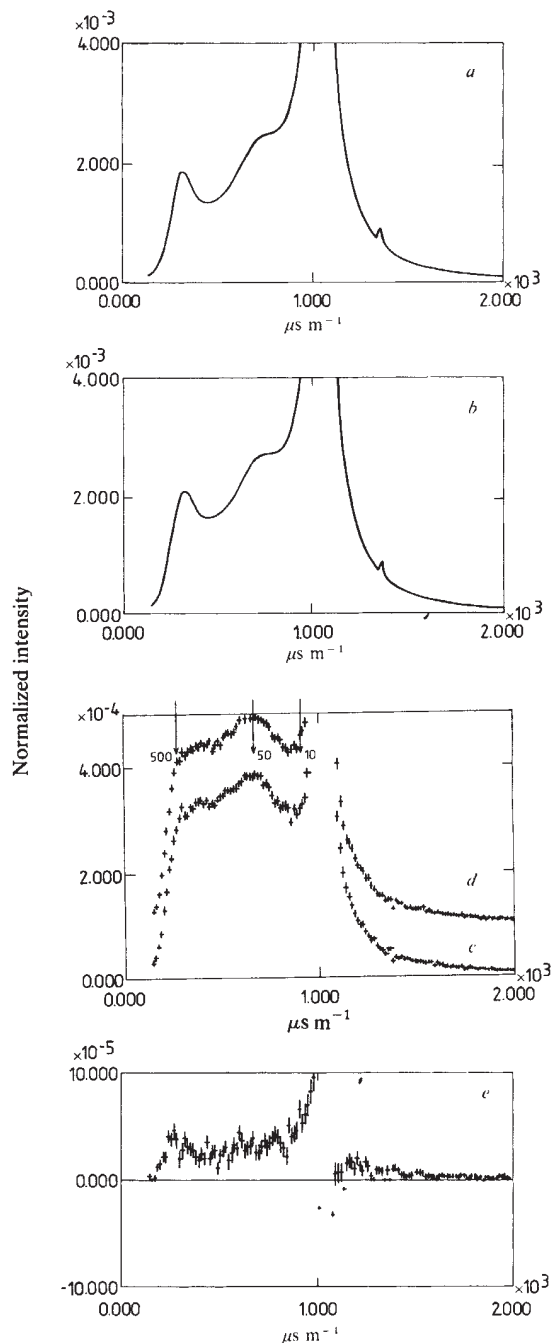


Fig. 2 Time-of-flight spectra measured at a mean angle of 97° of buffer (*a*) and hexokinase solution (*b*). The ordinates have been scaled by the elastic peaks measured with a vanadium sample. The abscissa is in time-of-flight (TOF) units ($\mu\text{s m}^{-1}$). Neutron energies are related to TOF by $E (\text{cm}^{-1}) = 42.2 \times 10^6 / (\text{TOF})^2$. The sharp peak on the energy loss side of both spectra is due to parasitic Bragg scattering from an aluminium window. Subtraction of the buffer (and quartz cell) scattering gives the corrected spectra of hexokinase (*c*) and hexokinase bound with glucose (*d*) (curve shifted by 10^{-4}). Energy transfers of 10, 50 and 500 cm^{-1} are indicated by arrows. The difference spectrum obtained by subtracting *c* from *d* is shown in *e*. The error bars are derived from the counting statistics and show 1 s.d. from the nominal value. Hexokinase (Sigma lot 120F-8080) in crystalline suspension was redissolved in D_2O buffer containing 20 mM Tris, 0.1 M NaCl, 0.1 mM dithiothreitol, 0.1 mM sodium azide at $p\text{D} (= p\text{H})$ 8.5 and dialysed exhaustively against the same buffer. 0.8 ml of solution (previously concentrated to 45 mg ml^{-1}) was then centrifuged at 16,000 r.p.m. for 5 min before being placed in a 1.5 mm thick quartz cell. The cell was set at 45° to the incident beam in the time-of-flight spectrometer and maintained at 15°C . The sample chamber was maintained at a slight over pressure of helium throughout the experiment to avoid parasitic air scattering. To saturate the protein with its substrate, 1.8 mg of glucose per ml of solution was added, the glucose being in 50% (by weight) solution in the same D_2O buffer. The unexchanged protons of glucose increase the number of protons in the solution by about 3%. Spectra were accumulated for about 36 h each.

modes is consistent with the expected scattering by harmonic oscillators⁷.

The dynamical modes of a protein should cover a wide range of different characteristic energies^{2,5}. Some will be very similar to those found in small molecules, for example C—C bending and stretching modes and rotation of methyl groups. Characteristic energies for such local modes will be in the range 100–2,000 cm⁻¹. Others are collective modes specific to the secondary structure of proteins, for example longitudinal acoustic modes of helices with energies in the range 25–200 cm⁻¹. At still lower energies (1–50 cm⁻¹) 'breathing' modes as found in globular elastic bodies should occur, although the nature of these may be significantly modified by solvent damping⁵. Some of these low frequency modes may be directly linked with protein function, for example, the postulated hinge-bending mode in enzymes having a substrate binding cleft¹⁵. The inelastic spectra that we have measured extend up to more than 500 cm⁻¹, confirming the expected broad frequency spread of protein motions.

There are small but distinct differences between the spectra given by the enzyme with and without substrate bound (see Fig. 2e). First, there is an overall reduction of 5–10% in the intensity of inelastic scattering on glucose binding. This difference could be due *a priori* to a reduced number of protons in ligated hexokinase (resulting from a higher degree of exchange) or to a genuine modification of the enzyme dynamics. The first hypothesis is unlikely as the number of exchangeable protons is relatively small (20% of all protons) and is likely to decrease rather than increase with the tightening associated with substrate binding. Furthermore, neither the intensity nor the width of the elastic peak changes on ligation, suggesting that the total number of protons is unaltered. Thus, we conclude that there is a real modification of the dynamics of hexokinase on binding glucose. That the difference spectrum is positive suggests that the ligated enzyme has fewer and/or smaller amplitude vibrational modes, in accordance with the view that ligation induces a stiffening of the enzyme structure.

Second, Fig. 2e clearly shows that the shape of the difference profile differs from that of the total inelastic scattering by hexokinase. Thus, while ligation appears to affect both local and collective modes, different frequencies are affected to different extents. In particular, the low frequency modes (<40 cm⁻¹) are more markedly affected, as was also found to be the case on binding an inhibitor to lysozyme⁹. The hinge-bending mode of lysozyme has been calculated¹⁵ to be between 5 and 15 cm⁻¹, and although no analogous calculations have been done for hexokinase, it is possible that the peak in the difference profile at 30 cm⁻¹ corresponds to the disappearance of such a mode on closing of the cleft around the glucose substrate.

Intramolecular vibrational modes contribute to the enthalpy and entropy of a protein and it has been proposed that changes in the vibrational spectrum may be important in the thermodynamics of protein reactions¹⁴. Our results suggest that there is a reduction in the vibrational enthalpy of hexokinase on glucose binding. However, it has been shown recently that there is practically no overall change of enthalpy on binding of glucose¹⁶. This is not inconsistent with our finding, as a reduction in the enthalpy of the vibrational modes could be compensating for the burying of a charged group. Indeed, we note that such a group (Asp 189) is reported to be removed from contact with the solvent as a result of the conformational change¹³. In future experiments we aim to extract from the variation with angle of the inelastic scattering spectra the frequency distribution of vibrational modes of the sample⁷. This would allow estimation of the change in the vibrational contributions to the entropy and enthalpy on ligation.

In this exploratory application of inelastic neutron scattering, we have established that it is now feasible to use measurements of the frequency spectrum of proteins in solution to characterize the change in dynamical state associated with binding a substrate molecule. Clearly, further experiments of this kind will

be needed to confirm the magnitude and significance of the observed changes. Several theoretical studies of protein fluctuations have been made by molecular dynamics simulations^{2,15,17,18} and normal mode analysis¹⁹, from which it is possible to predict inelastic neutron spectra. A more detailed interpretation of the inelastic neutron scattering spectrum of hexokinase would require careful comparison with the results of such calculations, although this is not yet practicable for such a large protein. Alternatively, one can envisage the use of selective labelling (for example, protonation of certain residues in an otherwise fully deuterated protein) to aid in assignment of particular modes. Thus, our results demonstrate the potential of inelastic neutron scattering for investigating the dynamic aspects of protein function and testing the theoretical understanding of protein dynamics.

Received 21 June; accepted 15 September 1982.

1. Careri, G., Fasella, P. & Gratton, E. A. *Rev. Biophys. Bioengng* **8**, 69–97 (1979).
2. Karplus, M. & McCammon, J. A. *CRC Crit. Rev. Biochem.* **9**, 293–349 (1981).
3. Frauenfelder, H., Petsko, G. A. & Tserrnoglou, D. *Nature* **280**, 558–563 (1979).
4. Rosa, J. J. & Richards, F. M. *J. molec. Biol.* **133**, 399–416 (1979).
5. Petricolas, W. L. *Meth. Enzym.* **61**, 421–458 (1978).
6. Lovesey, S. W. & Springer, T. (eds) *Dynamics of Solids and Liquids by Neutron Scattering* (Springer, Berlin, 1977).
7. Boutin, H. & Yip, S. *Molecular Spectroscopy with Neutrons* (MIT Press, 1968).
8. *Neutron Beam Facilities Available for Users* (Institut Laue-Langevin, Grenoble, 1981).
9. Bartunik, H. D., Jolles, P., Berthou, J. & Dianoux, A. J. *Biopolymers* **21**, 43–50 (1982).
10. Anderson, C. M., Stenkamp, R. E. & Steitz, T. A. *J. molec. Biol.* **123**, 15–33 (1978).
11. Bennett, W. S. & Steitz, T. A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4848–4852 (1978).
12. Bennett, W. S. & Steitz, T. A. *J. molec. Biol.* **140**, 211–230 (1980).
13. McDonald, R. C., Steitz, T. A. & Engelman, D. M. *Biochemistry* **18**, 338–342 (1979).
14. Sturtevant, J. M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2236–2240 (1977).
15. McCammon, J. A., Gelin, B. R., Karplus, M. & Wolynes, P. G. *Nature* **262**, 325–326 (1976).
16. Takahashi, K., Casey, J. L. & Sturtevant, J. M. *Biochemistry* **20**, 4693–4697 (1981).
17. McCammon, J. A., Gelin, B. R. & Karplus, M. *Nature* **267**, 585–590 (1977).
18. Karplus, M. & McCammon, J. A. *Nature* **277**, 578 (1979).
19. Tasumi, M., Takeuchi, H., Ataka, S., Dwivedi, A. M. & Krimm, S. *Biopolymers* **21**, 713 (1982).

Errata

In the letter 'Pluripotent embryonic stem cell lines can be derived from t^{w5}/t^{w5} blastocysts' by T. Magnuson *et al.*, *Nature* **298**, 750–753 (1982), in Fig. 2 the lowest bands on the gels should be labelled 17 (kilobases). In line 3 of Fig. 2 legend $129(t^0/t^{w5})$ is incorrect and should read $129(t^0/t^{w125})$.

In the letter 'Potent new inhibitors of human renin' by M. Szelke *et al.*, *Nature* **299**, 555–557 (1982), structure 4 in Table 1 erroneously contains the superscript R over the Phe-Phe peptide bond.

Corrigenda

In the letter 'Direct imaging of travelling Rayleigh waves by stroboscopic X-ray topography' by R. W. Whatmore *et al.*, *Nature* **299**, 44–46 (1982), the references to 'half-wavelength' or ' $\pi/2$ ' in Fig. 4 legend and in the relevant discussion in the text should read 'one wavelength' or ' π '.

In the News and Views article 'Monoclonal antibodies to human malignant melanoma' by R. A. Reisfeld, *Nature* **298**, 325–326 (1982), the names of two individuals who contributed considerably to the work described were omitted: Drs K. O. Lloyd and L. J. Old of the Memorial Sloan Kettering Cancer Center, New York.

The following note relates to the letter 'Do graded units of accretionary spheroids from the Barberton Greenstone Belt indicate Archaean deep water environment?' by I. G. Stanistreet *et al.*, *Nature* **293**, 280–284 (1981). Interested readers are referred to the work of T. Heinrichs, Lithostratigraphische Untersuchungen in der Fig Tree Gruppe des Barberton Greenstone Belt zwischen Umsoli und Lomati, *Göttinger Arb. Geol. Paläont.* **22**, 119pp (1980), which was not available to the authors during the preparation of the above manuscript.

BOOK REVIEWS

Ruskin then, science now

Michael Rodgers

COMPANY histories are not written primarily for the general reader and this one, celebrating 175 years of publishing at Wiley, is no exception. Nevertheless there is much here that those interested in books and publishing will appreciate.

In addition to journals, professional, research and reference works, Wiley's list contains many well-known science textbooks. Tracing the evolution of that particular species, the college textbook, provides the most interesting strand of this story, beginning in the 1940s when the United States government enacted its GI Bill of Rights. This entitled veterans of the Second World War to educational assistance, thus boosting the demand for college textbooks that had begun in the early 1940s, which in its turn arose from the armed services' large-scale provision of technical training. The bonanza for American textbook publishers came to an abrupt end in 1948, and not simply because the last of the ex-GIs had completed their college education. Publishers then were not over-concerned with such details as planning, cost accounting, and pricing policies; escalating costs were not being passed on to their customers. The boom fostered complacency, since little effort was required to satisfy students prepared to put up with dull, old-fashioned textbooks; their successors straight from school were more particular. A difficult period of adjustment lay ahead: a familiar pattern to those who, like me, entered publishing (in the UK) at the tail-end of the golden age of the 1960s.

For John Wiley & Sons there was the added irony that they had been steadily building a list of impressive titles in corporate management, yet they seemed unable to put this body of wisdom into practice. Their own solution to the general problem besetting the industry was to enrol four executives in the Harvard Business School's Advanced Management Programme in 1953. By the end of that year the company president, Bradford Wiley, was himself at Harvard to study modern business methods, returning

enthusiastic, business-oriented, hardnosed, and determined to concentrate his full energies on the financial aspects of publishing.

No doubt it was an interesting time for Wiley employees as the new brooms returned.

Textbooks in the early days were the result of the buckshot approach: the publication of books with a broad appeal that *might* be adapted to specific course

Wiley: One Hundred and Seventy five Years of Publishing. By John Hammond Moore. Pp.279. ISBN 0-471-86082-4. (Wiley: 1982.) £14.75, \$25.

needs. In the 1950s the search for the customer still began after a textbook had been produced. Andrew Ford, a Wiley traveller of that era, notes that

Editors relied on advisers, series editors, consultants, boards, and so on. Just look at our catalogs of those years. Nothing but one series after another.

This may produce a wry smile from some of my contemporaries in British publishing: we started out doing much the same thing, though 20 years later.

Ford, brought into the office as a junior editor in 1957, found

an academic-like atmosphere where chief editors kept top schools to themselves like prizes for seniority and only grudgingly gave approval to manuscripts procured by a new employee.

The vital role of basic introductory textbooks in providing a reliable — and substantial — backlist income was yet to be fully appreciated. This was to change, and the 1960s saw a striking increase in Wiley's share of the college market. In 1961 their list contained 18 college titles with annual sales of 10,000 copies or more; in 1971 the figure had risen to 46.

How do the really successful textbooks actually begin life? It is a chancy business, for all the talk of planning, with the right author and the right editor coming together just when the market is ready for what they will eventually develop. We are given a fascinating glimpse of the genesis of Halliday and Resnick's *Physics for Students of Science and Engineering*, one of the most famous of science textbooks. Towards the end of 1954 one of Wiley's college editors, Robert B. Polhemus, happened to be visiting Resnick at the University of Pittsburg. A number of publishers had been urging Resnick and his departmental colleague Halliday to turn their lecture notes into a textbook. Polhemus noted this in his report to the New York office, continuing:

The idea has blown hot and cold. Right now it's cold, and Halliday told me afterwards that he has definitely decided against doing such a book.

But they did and the rest, as they say, is history. Published in 1960, the parent text and its various abridgements and supplements have sold almost two million copies.

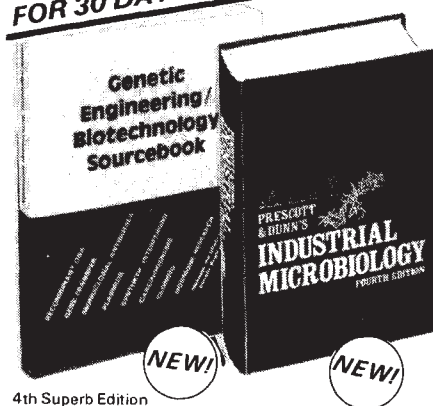
One year later Wiley merged with Interscience Publishers, the creation of two Europeans, Maurits Dekker and Eric S. Proskauer. Their original aim had been to provide a European-style publishing house in New York for émigré scientists in America. When the firm was established in 1940, German chemistry texts were used throughout the world: one of the first Interscience titles was an English translation of Karrer's *Organic Chemistry*. Thirty years later roles had been reversed, with the translation of Cotton and Wilkinson's *Inorganic Chemistry* becoming the standard text in German universities.

The predictable question concerning Wiley's future is posed on the penultimate page. In the 1980s they expect more expansion in the professional, medical and international groups than in the educational group: a reflection of an increasing demand for technical skills in the Third World coupled with a stable, or declining, college population in the developed countries. Of course it is the electronic revolution that will affect the company's long-term prospects, and although there are no specific forecasts, companies such as Wiley will doubtless have no real trouble in coping with the impact of electronics on reference sources generally. The best textbooks are a different matter. The experience of reading them could not be created by resorting to a screen, and their development will always need individuals. It is easy for Wiley to maintain that a future with an increasingly professional management, executives who refer to books as "product", sophisticated market research, and all the rest, will not stifle individual enterprise, but we look to their next decades' activity to prove the point.

Wiley's birthday volume is written in an earnest, respectful style, as befits an official history, but there are plenty of good stories and anecdotes about the life and times of a company that once published James Fenimore Cooper and John Ruskin. One appealing story concerns a railroad fan, Andrew Neilly, who joined Wiley as a traveller in order to see the nation by train, and for two years (1947–1949) he did just that. It must have been fun. And good background too, for he ended up as company president. □

Michael Rodgers, formerly with Oxford University Press and W.H. Freeman, is joining Longmans after a brief spell at Nature.

**SPECIAL
nature SUBSCRIBERS
OFFER-EXAMINE ON-APPROVAL
FOR 30 DAYS WITHOUT RISK**



4th Superb Edition
Prescott & Dunn's

Industrial Microbiology

Edited by Gerald Reed
Vice President, Amber Laboratories USA

The up-to-date guide and reference tool to industrial technology and applications in the microbiology of foods and food ingredients — with a special section on biological production of fuel alcohol.

Macmillan Hardcover 896 pp £42.50
ISBN 0 333 33630 5 1982

Genetic Engineering/ Biotechnology Sourcebook

Never before published! Profiles of 1,529 recent and ongoing genetic engineering/biotechnology research projects funded by 122 US government and non-profit organizations.

Covers research affecting: ★ Pharmaceuticals ★ Medicine and Diagnosis ★ Agriculture ★ Food Processing ★ Energy ★ Chemicals.

Reveals for each project ■ Nature and objectives of research ■ Funding source and money awarded ■ Names and addresses of individual researchers.

Special Yellow Pages reference section — tells you at a glance who's researching what, where, when and how.

Macmillan Softcover 350 pp £52.50
ISBN 0 333 34148 1 1982

Place your on-approval order today!

GLOBE
BOOK SERVICES LTD

Canada Road, Byfleet, Surrey KT14 7JL, England.
Tel: Byfleet (09323) 40397.

A MEMBER COMPANY OF MACMILLAN PUBLISHERS LTD.

EXAMINE ON 30 DAYS APPROVAL

To: Ian Jacobs, Globe Book Services Ltd., FREEPOST,
Canada Road, Byfleet, Surrey KT14 7BR, England.

(Please tick boxes required)

Please send me _____ copies of ☐ *Genetic Engineering/Biotechnology Sourcebook* at £52.50 each plus P&P (UK £2, Overseas £3) and/or _____ copies of ☐ *Industrial Microbiology* at £42.50 each, plus P&P (UK £2, Overseas £3) on 30 days approval. If I am not completely satisfied, I may return the volume(s) within that period for a full refund.

Tick preferred method of payment

☐ I enclose cheque for £ _____

☐ Charge my credit card £ _____

☐ Visa ☐ Access ☐ American Express ☐ Diners

Card No. _____ Expiry date _____

☐ Bill me. I need send no money now.

Signature _____
(Orders cannot be accepted without a signature)

Name _____ (PLEASE PRINT)

Address _____

Postcode _____

Allow 28 days for delivery
POST TODAY - NO STAMP NEEDED

The protocol for arranging nature

Michael B. Usher

Multivariate Analysis in Community Ecology. By Hugh G. Gauch Jr. Pp.298. Hbk ISBN 0-521-23820-X; pbk ISBN 0-521-28240-3. (Cambridge University Press: 1982.) Hbk £20, \$37.50; pbk £7.95, \$14.95.

ECOLOGISTS collect multivariate data. Very rapidly the number of observations, each being the occurrence or abundance of one of a large number of species in one of a large number of quadrats, baffles the observer, who needs some kind of protocol for arranging and viewing the data. Multivariate analyses provide that protocol, but the diversity of possible methodologies can confuse all but the most numerate of ecologists.

In this new book, Hugh Gauch attempts to explain the methodology of multivariate analysis without delving into the mathematical or computational complexities. The introductory chapter deals with hand sorting: the sceptic only needs to look at Table 1.2 with 50 species recorded from 25 quadrats to see how even a small data set can look incomprehensible until it is re-arranged, as in Table 1.3.

Gauch makes the point that more objective methods for arranging all of the individual observations are required, and he devotes the remainder of the book to discussion of the three basic approaches to sorting ecological data of this type — direct gradient analysis, ordination and classification. Although the structure of each chapter differs, generally the rationale of the approach is described, the methods are explained in words rather than in mathematical symbols, a number of examples are quoted (illustrations lead to a geometrical interpretation of the results), some of the known problems are described, and finally there is a broad evaluation. It is these chapters which give the book its value.

There are two drawbacks, however, that prevent this being a complete textbook. First, if anyone actually wishes to undertake an analysis they will have to turn to another book for the recipe. True, there is a two-page appendix included on computer programs, but this is too brief to be a usable consumer's guide. Second, the text is very Cornell-orientated — perhaps not surprisingly, since Gauch is closely associated with the group which worked with the late R. H. Whittaker at that university. An example is the "horseshoe" problem, where their DECORANA method of tackling it is described but others are not considered (though to be fair the extensive catalogue of applications in Chapter 6 partly overcomes this problem).

Nonetheless, the clear explanation and the comparisons between the various approaches make the book a suitable text for the student or researcher who wishes —

or has — to understand this field of quantitative ecology. As the author in general cleverly avoids the mathematical complications, the apparent simplicity may be beguiling. But I still plan to use this book as a background text when teaching multivariate methods. □

Michael B. Usher is a Senior Lecturer in the Department of Biology at the University of York.

Teaching machines

Margaret Boden

Intelligent Tutoring Systems. Edited by D. Sleeman and J.S. Brown. Pp.345. Hbk ISBN 0-12-648680-8. (Academic: 1982.) £19.20, \$39.50.

INTELLIGENT tutoring systems (ITSs) are programmed teaching-aids grounded in artificial intelligence (AI). They differ radically from familiar types of computer-assisted-instruction (CAI). Many of the latter merely present repetitive "drill-and-practice" exercises; others can vary their response with the student's level of understanding by means of branched programs with predefined choice-points, but only to a very limited degree. ITSs are considerably more flexible, and better able to adapt to the idiosyncracies of a student's thinking.

This flexibility derives from the fact that ITSs contain complex computational models of students' knowledge and thinking. These control the questions and responses that an ITS presents to the student, and generate its diagnoses of students' mistakes. Unlike traditional CAI, the emphasis of ITSs is less on facts than on ways of thinking about a problem-domain; they function not so much by presenting "correct" examples of solutions as by providing relevant hints or questions, and — significantly — by diagnosing the thought-strategies behind the student's mistakes so as to guide the tutorial response.

Such diagnosis requires an understanding (in explicit, programmed, form) of the variations in cognitive structure and inference that underlie different sorts of (non-random) mistake. Computer science has a word for such mistakes — "bugs". A bug is a precisely definable and relatively systematic erroneous variation of a correct procedure (this definition, though, optimistically implies an underlying correctness). Much work on ITSs is focused on identifying and using bugs in the student's thinking.

For example, DEBUGGY is a program that is better than human beings at diag-

nosing the (nearly one hundred) different bugs that can explain a student's errors in doing subtraction. Its authors have provided a precise notation for describing these, and are also developing a "generative" theory of bugs — that is, one that can be applied to a particular (correct) procedural skill to generate all the bugs actually observed, and no others. This theory aims also to explain the pattern of bugs observed, so that (for example) two superficially different mistakes in subtraction can be understood as two variations of the same underlying difficulty in the student's mind. The ITS thus embodies a psychological theory predicting the detailed errors observed during learning.

But this program, like most of the other ITSs described in Sleeman and Brown's volume, could not be let loose in our schools tomorrow. This is partly because its psychological theory (like all current theories of learning) is inadequate to capture the full complexity of learning, and partly because AI cannot yet provide a "friendly" computing environment such that an untrained person (child or teacher) can use it. Given a specially-trained teacher, however, it can be used in the classroom to help identify precisely what is going awry in the children's thinking.

The complexity of the computational models within ITSs is such that they deal only with limited topics, as opposed to entire curricular courses (a further contrast between ITSs and CAI). Examples described in the book (which is edited by two pioneers in the field) include medical diagnosis of specific kinds, faults in electronic circuits, interpretation of magnetic resonance spectra and various mathematical topics. But the editors suggest that many of these forms of thinking are generally useful outside these particular applications — perhaps ITSs are the new Latin?

Sleeman and Brown's useful collection summarizes the state-of-the-art, and highlights problems as well as promises. They admit that current ITSs are excessively *ad hoc*, reflecting unprincipled intuitions about how to control their behaviour rather than well-founded psychological and computational theories about teaching-learning strategies. As well as improvements in AI techniques (for handling natural language dialogue, for example) we need more experimental investigation of how to transform students' misconceptions into constructive learning experiences. But ITSs could help direct the relevant research. One can thus endorse the editors' words:

We hope that educational theorists will find the explicit formulation of tutoring, explanation and diagnostic processes inherent in ITSs a test bed for developing more precise theories of teaching and learning. □

Margaret A. Boden is Professor of Philosophy and Psychology at the University of Sussex.

Inside cytoplasm for outsiders

Harriet Harris

Organization of the Cytoplasm, Parts 1 and 2. *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. XLVI. Pp.1,047. ISBN 0-87969-045-3. (Cold Spring Harbor Laboratory: 1982.) \$130 (US), \$156 (elsewhere).

ONE of the principal goals of cell biology is to describe the functions and behaviour of the living cell in terms of its molecular components. Even with the objective restricted to an understanding of the cytoplasm, however, the complexity of function and the number of constituent organelles and macromolecular components is daunting. Nonetheless the progress with certain specialized systems (such as eukaryotic flagellae or intestinal microvilli) towards the correlation of biochemistry, structural organization and dynamic function gives hope that the ultimate goal can be achieved.

The symposium on which these volumes are based brought together a wide diversity of topics, ranging from properties of bulk cytoplasm to details of molecular interactions. It would be fair to comment that relatively few of the contributors to the books might be described as studying cytoplasm *per se*, and it appears that one aim of the two-volume work is to stimulate a sense of common purpose between researchers in fields as distinct as, for example, membranes and microtubules.

The behaviour of living cytoplasm must provide the essential framework within which to view both ultrastructural and biochemical results. This field is being greatly stimulated by new techniques in light microscopy, sometimes in combination with the microinjection of foreign substances into the cytoplasm. These yield information on the movement of macromolecules and organelles through the cytoplasm, or may detect changes in important regulatory factors such as pH and calcium ion concentration. Such developments are still in their infancy, but offer the exciting possibility of enabling us to describe actual events in the cytoplasm, which is a vital step on the way to elucidating by what mechanisms such events occur.

A fruitful approach to mapping the spatial organization of the cytoplasm has been to localize known proteins within fixed cells by means of specific antibodies, for example, by immunofluorescence. Such techniques have been nowhere more productive than in the extensive characterization of three sets of cytoskeletal filaments — microtubules, intermediate (100Å) filaments and microfilaments — all of which are well represented in these volumes. A description of the location and composition of a structure, however, is not necessarily the key to comprehending its function, and an

outstanding question on the cytoskeleton concerns the role of intermediate filaments. This problem is being tackled by microinjection of filament-specific antibodies to selectively dissociate the networks within living cells, an experimental approach which holds promise as a general method for probing elusive functions. Often the most useful data on the static organization of macromolecules are obtained with simplified systems. Two striking examples are the packing of actin filaments in stereocilia and the linkage of the cytoskeleton to membranes in erythrocytes, both of which provide general models applicable to less specialized cell types.

Another theme of the symposium, reflected in the books, concerned the mechanisms by which cytoplasmic order can be maintained in complex, multi-component, dynamic systems. This is a speculative area and fertile ground for hypothetical models, but some highly productive experimental systems are also described. Protein-sorting problems are discussed by several authors, in a variety of contexts; these include selective insertion of proteins into or through membranes on synthesis, synthesis and expression of cell surface or secretory proteins, and selective uptake at the surface, all processes which may occur rapidly with continuous turnover and where high specificity is essential to deliver the proteins to their correct destinations. Other cytoplasmic components which may be in a highly dynamic state are the microfilaments and microtubules, where these form transient assemblies, such as the mitotic spindle. Assembly must be under rigorous control, since the correct location and polarity of filaments is essential in determining function. Current data indicate that order may be achieved by discrete organizing centres for filament growth, but these would only be effective in conjunction with the suppression of spontaneous, unregulated filament formation. A wealth of data from *in vitro* biochemical studies of filaments and their associated proteins generally support such a model, but proof of the effective modes of control *in vivo* will be more difficult to achieve.

What are the physiological switches signalling changes in cytoplasmic behaviour? Calcium, acting both directly and by calmodulin-mediated processes, including phosphorylation, is heavily implicated, as are cyclic AMP-dependent and other kinases. The space devoted to regulation in the books is disappointingly small, perhaps because the areas of ignorance are still too large for meaningful questions to be asked.

The attempt of the editors to unite a range of subjects under the single heading of cytoplasmic organization is timely, but

not wholly successful. Fully comprehensive coverage of such a large field is not to be expected, even in a thousand pages, but the balance between different topics is in places very uneven. Only a handful of articles, for example, make any reference to the soluble components of the cytoplasm, but nearly half of the books (surely too much) is devoted to cytoplasmic fila-

ments. These volumes do however contain a wealth of interesting material, while the breadth of ground covered makes them especially valuable to more general readers, or to cell biologists exploring areas peripheral to their own. □

Harriet Harris is at the Laboratory of Molecular Biology, Cambridge, UK.

Tectonics: moving with the times

Janet V. Watson

Plate Tectonics and Crustal Evolution, 2nd Edn. By Kent C. Condie. Pp.310. Hbk ISBN 0-08-028076-5; pbk ISBN 0-08-028075-7. (Pergamon: 1982.) Hbk £32.50, \$65; pbk £13.10, \$29.50.

SO MANY books have been written in the aftermath of the plate tectonic upheaval that any new arrival is bound to encounter some resistance. Kent Condie's volume is a second edition, issued six years after the original, and can therefore be said to have already justified its existence.

Has the new edition moved with the times? Although text, figures and tectonic map have been updated with respect to many details, my impression is that the book retains not only the merits but also the defects of its predecessor. Kent Condie is distinguished for his work on Precambrian magmatic assemblages, and his treatment of the Precambrian magmatic provinces and of igneous phenomena generally is thorough and effective. He is successful in showing how geophysical and geochemical data can contribute to the understanding of geological problems and he has been unusually courageous in his attempts to elicit a general picture from the scrappy and often contradictory evidence about the deep structure of the crust and mantle. The numbers assigned to such values as the average thicknesses of crust in different types of province make good Aunt Sallys for the knowledgeable, though the less-experienced reader might have been better served if a range rather than an average had been quoted.

The inequalities of standard of treatment seem more apparent in this second edition than they did in 1976. In particular the disparity between the sophisticated discussion of magmatic phenomena and the cursory treatment of sedimentary basins has become more glaring. The author justifies the omissions on the reasonable grounds of lack of space and on the need to avoid overlap with other published works. Nonetheless, plate tectonics without the sedimentation story is Hamlet with no more than half the Prince of Denmark.

I have one final personal regret which concerns the otherwise welcome world tectonic map which is contained in a pocket at the back of the book. Maps are not only valuable sources of information, they can also be objects of beauty that kindle the imagination and stimulate the initiated and the expert alike; this one succeeds in its first role but is sadly deficient in the second. □

Janet Watson is a Professor in the Department of Geology at Imperial College, University of London.

All of the Araneae pulled together

J.L. Cloudsley-Thompson

Biology of Spiders. By R.F. Foelix. Pp.306. ISBN 0-674-07431-9. (Harvard University Press: 1982.) \$35, £21.

ALTHOUGH there are some recent and quite excellent books on the ecology and natural history of arachnids in general and of spiders in particular, no new attempt has been made for over half a century to provide a comprehensive synthesis of the general biology of these fascinating creatures. The book under review, the original version of which appeared in 1979 as *Biologie der Spinnen* (published by Georg Thieme Verlag, Stuttgart), is an attempt to pull together the various facets of a spider's life. It is not a condensed handbook or reference book so much as

an advanced introduction to the group.

Spiders are found throughout the world and, according to Rainer Foelix, "have conquered all ecological environments, with perhaps the single exception of the air": and, one might add, of the seas. The biology of freshwater spiders (*Argyroneta aquatica*, *Dolomedes* spp. etc.) is discussed in some detail, but little mention has been made of marine littoral spiders, although these do occur as well. It is, in truth, no exaggeration to say that all possible ecological niches on land have been occupied, including rather barren environments such as sand dunes, tidal zones or mountain tops. The distribution of spiders, extending even to oceanic islands, can be explained by the ability of the young of many species as well as of adult Linyphiidae, to float through the air, ballooning on their own threads. That spiders cannot bridge really large distances by aeronautical means, however, is indicated by the fact that East Africa and Madagascar are only 400 km apart, yet the spider fauna of each is quite different.

The interactions between spiders and their environments have been investigated systematically only within the past few decades. During this time, however, information about the ecology of spiders has burgeoned to such an extent that only a few selected themes have been discussed in the chapter on ecology. These include distribution, habitats, prey, enemies, thermoregulation, overwintering, camouflage and mimicry, communication and social behaviour. Other chapters are concerned with functional anatomy, metabolism, neurobiology, spider webs, locomotion and prey capture, reproduction, development, phylogeny and systematics.

The book is well illustrated with photographs and line drawings. It is interestingly written, and the author has managed to convey to the reader some of his own obvious enthusiasm for the subject. He cannot fail to have engendered further interest in spiders, thereby militating against the neglect they have so long been accorded and which he himself so deplores.

J.L. Cloudsley-Thompson is Professor of Zoology at Birkbeck College, University of London.

NEURO-TRANSMITTER AGONISTS FROM BIOSEARCH

BIOSEARCH offers a number of unique neurotransmitter agonists of recognized value. All are natural products isolated in our laboratories and are of exceeding purity.

QUISQUALIC ACID
416x potency of glutamate
\$60/10mg \$300/50mg
\$500/100mg

IBOTENIC ACID
22x potency of glutamate
\$60/10mg \$300/50mg
\$500/100mg

Inquire about additional glutamate and GABA agonists as well as other BIOPERFECT natural products.



BIOSEARCH

1281-F Andersen Dr.
San Rafael, CA
USA 94901
Tel. 415 459-3907
Telex 172 029BIOSEARCH

Circle No.59 on Reader Service Card.

ANNOUNCEMENTS

Awards

The 1982 Kone Award has been awarded by the Association of Clinical Biochemists to **Professor Poul Astrup** (Copenhagen) for his contribution to clinical chemistry.

The winners of Columbia University's 1982 Louisa Gross Horwitz Prize are **Barbara McClintock** (Carnegie Institution's Genetic Research Unit at Cold Spring Harbor) and **Susumu Tonegawa** (Center for Cancer Research and the Department of Biology at the Massachusetts Institute of Technology).

The Lita Annenberg Hazen Award and Fellowship Grant aims to recognize major achievement and superior potential and to foster a greater commitment by physicians to clinical research. A prize of \$50,000 (tax free) will be awarded to the physician-investigator or team of physicians for current and published work, that is of evident superiority and importance. A companion grant of \$50,000 will be given to the investigator's institution for the support of a Research Fellow or Fellows whom the Award winner will select as associates. Further details from Mount Sinai School of Medicine of the City University of New York, Office of the Dean, 1 Gustave L. Levy Place, New York, New York 10029, USA.

Applications for training fellowships in 1983-84 are invited from junior scientists wishing to be trained in those aspects of cancer research related to the International Agency for Research on Cancer's programme: epidemiology, biostatistics and environmental carcinogenesis — both chemical and viral. Applicants should be engaged in research in medical or allied sciences and intend to pursue a career in cancer research. Fellowships are awarded for one year and are tenable at the Agency or in another suitable institution abroad. Further details from: the Head of the Research Training and Liaison Programme, International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon Cedex 08, France.

The National Physical Laboratory is to set up an Awards Scheme aimed at furthering knowledge and understanding of metrology. In 1983 single-payment awards of up to £5,000 will be made to individual scientists on the full-time academic staff of UK universities and polytechnics, who submit the best ideas and proposals for research and development work likely to lead to a major advancement in measurement. Applications should reach Dr K.C. Shotton, Secretary, NPL Metrology Awards Scheme, National Physical Laboratory, Teddington, Middlesex, UK, by 31 December 1982.

The Institution of Mechanical Engineers and Esso announce the Tribology Award 1982/83. A £300 prize will be given for an essay on industrial aspects of lubrication, friction or wear, submitted by 31 March 1983. Anyone under 35 engaged in industry or on a sandwich course may enter. Further details from: Mr J.H. Cole, Divisions Co-ordinator, The Institution of Mechanical Engineers, 1 Birdcage Walk, Westminster, London SW1, UK.

If you work in a university outside the UK, or in a polytechnic, college or school anywhere and your work is held up for lack of modest funding, the RSC Research Fund may be able to help you. Further details from: Mrs E.S. Wellingham, The Royal Society of Chemistry, Burlington House, London W1, UK.

Appointments

Mr Roland List (Canada) has been appointed Deputy Secretary General of the World Meteorological Organization (WMO) succeeding Raymond Jean Schneider (Switzerland) who has retired.

The new board of governors of the International Atomic Energy (IAEA) have elected the governor from Czechoslovakia, **Ambassador Emil Kebluser** to be Chairman from 1982 to 1983. The vice-chairmen are: **Luigi Noe** (Italy) and **Adolfo R. Taylhardat** (Venezuela). **Bernard Bechetoille** (France) is the new director of the Division of Budget and Finance.

The following appointments have been made within CSIRO: **Dr David Smiles** has become chief of the Division of Soils, based in Canberra; **Dr Don Gibson** is chief of the Division of Energy Technology in Melbourne; **Dr David Mitchell** is officer in charge of the Centre for Irrigation Research at Griffith in New South Wales; **Dr Alan Reid** is chief of the Division of Mineral Engineering in Melbourne.

Sir Keith Joseph, Secretary of State for Education and Science, has appointed three new members to the Natural Environment Research Council: **Lord Cranbrook**, **Professor Ronald Edwards** and **Professor John Simpson**. Reappointed are **Professor William Stewart**, **Mr Hugh Fish** and **Professor Bernard Leake**.

J. Mark Danley has been appointed Group Leader for Education and Training at BioSciences Information Service (BIOSIS).

Dr I. Kelsey Fry is the new president of the British Institute of Radiology 1982-83.

Mr John Banks has been appointed president of the Institution of Electrical Engineers (IEE). The retiring President is Sir Francis Tombs.

Dr Donald J. Reis has been chosen as the first George C. Cotzias Distinguished Professor of Neurology at Cornell University Medical College.

The French Minister of National Education has conferred the title of Doctor Honoris Causa of the University of Nancy on **Prof. A.R. Ubbelohde** (Imperial College, University of London).

The governing body of the Rowett Research Institute has appointed **Dr Philip James** to the post of director in succession to Sir Kenneth Blaxter.

Derek M. Lambert (Cyril Blumfield & Partners) has been appointed president of the Concrete Society.

Martin C. Steele, formerly in charge of the semiconductor electronics group at the General Motors Research Laboratory, has been appointed acting director of the Institute for Amorphous Studies, Michigan.

George A.M. Cross, formerly at the Wellcome Research Laboratories (Beckenham, UK) has been appointed a professor at The Rockefeller University to head a new laboratory of molecular parasitology.

Dr C.W. Davidson (Department of Electrical and Electronic Engineering at Heriot-Watt University) is the new chairman of the Institution of Electrical Engineers' Science, Education and Technology Division.

Meetings

7 December, **Plant Cell Culture**, London (S. Whittaker, Oyez Scientific and Technical Services Ltd, Bath House, Holborn Viaduct, London EC1, UK).

15-17 December, **Annual Meeting of the British Medical Ultrasound Society**, Sheffield (British Institute of Radiology, Institute House, 36 Portland Place, London W1, UK).

20-22 December, **Biochemistry of Carcinogenesis**, London (The Biochemical Society, 7 Warwick Court, High Holborn, London WC1, UK).

20-22 December, **The Detection and Measurement of Hazardous Substances in the Atmosphere**, London (Dr J.F. Gibson, The Royal Society of Chemistry, Burlington House, London W1, UK).

10-13 January 1983, **Development of the Vascular System**, London (The Ciba Foundation, 41 Portland Place, London W1, UK).

17-21 January, **CIMO Working Group on Upper-Air Technology Basic to User Needs**, Geneva (World Weather Watch Department, World Meteorological Office, Geneva, Switzerland).

25 January, **Radiological Protection — Future Research Requirements**, London (Prof. J.H. Martin, Dept of Medical Biophysics, Blackness Laboratory, University of Dundee, Dundee, UK).

7-11 February, **Water Hyacinth**, Hyderabad (Dr G. Thyagerajan, Regional Research Laboratory, Hyderabad 500 009, India).

13-18 February, **Recent Advances in Bone Marrow Transplantation**, Utah (UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024, USA).

16 February, **New Radiopharmaceuticals and their Clinical Applications**, London (The British Institute of Radiology, Institute House, 36 Portland Place, London W1, UK).

21-23 February, **Diet and Nutrition**, Tel Aviv (The Secretariat, International Conference on Diet and Nutrition, PO Box 29784, Tel Aviv, 61297, Israel).

21-25 February, **Cooperative Education**, Melbourne (The Chairman, World Conference on Cooperative Education, PO Box 218, Hawthorn, Victoria 3122, Australia).

1-4 March, **Space Physiology**, Toulouse (CNES, Department des Affaires Universitaires, 18 Ave Edouard-Belin, 31055 Toulouse Cedex, France).

21 February — 4 March, **Commission for Agricultural Meteorology**, Geneva (World Climate Programme Department, World Meteorological Organization, Geneva, Switzerland).

7-12 March, **Analytical Chemistry and Applied Spectroscopy**, Atlantic City (Pittsburgh Conference, 437 Donald Rd, Pittsburgh, Pennsylvania 15235, USA).

8-11 March, **Nickel in the Human Environment**, Lyon (Dr W. Davis, IARC, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France).

9 March, **Digital Radiography**, London (The British Institute of Radiology, Institute House, 36 Portland Place, London W1, UK).

13-17 March, **Microolithography**, California (SPIE, PO Box 10, Bellingham, Washington 98227-0010, USA).

14-16 March, **Biomedical Research**, Heidelberg (Charles River Foundation, PO Box 430, Wilmington, Massachusetts 01887, USA).

14-17 March, **Microbiological Quality Assurance in Industry**, Brighton (Scientific Symposia Ltd, 33-35 Bowling Green Lane, London EC1, UK).

14-17 March, **Poliomyelitis Control**, Washington DC (Dr E.C. Chamberlayne, Fogarty International Center, Building 16A Room 205, National Institutes of Health, Bethesda, Maryland 20205, USA).
14-18 March, **Reactions and Intermediates in Nitrogen Fixation Processes**, Brighton (EUCHEM Conference, ARC Unit of Nitrogen Fixation, University of Sussex, Brighton, UK).

14-18 March, **Informal Coordination Meeting on the Use of INMARSAT**, Geneva (World Weather Watch Department, World Meteorological Organization, Geneva, Switzerland).

27-30 March, **1st European Symposium on Radiopharmacy and Radiopharmaceuticals**, Elsnore (Isotope Pharmacy, Frederikssundsvej 378, DK 2700 Copenhagen/Bronsho, Denmark).

21-25 March, **Annual American Society for Neurochemistry Meeting**, Honolulu (Dr G.R. Dutton, College of Medicine, University of Iowa, Iowa 52242, USA).

25-28 March, **British Society for Cell Biology**, Liverpool (Local Meeting Secretary, Dept of Medical Cell Biology, Liverpool University, UK).

26 March-1st April, **Gene Expression**, Park City (UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024, USA).

New ventures

The Arab Bureau of Education for the Gulf States has started publishing a new journal, entitled *The Arab Gulf Journal of Scientific Research* which will be devoted to research in basic and applied sciences. All communications should be directed to: Managing Editor, Arab Bureau of Education for the Gulf States, PO Box 3908, Riyadh, Saudi Arabia.

During the 18th General Assembly of the International Astronomical Union, a new IAU commission with the title: 'Commission 51 — Search for Extraterrestrial Life' was approved. The objectives will include: the search for planets around other stars; the search for radio transmissions, intentional or unintentional, of extraterrestrial origin; the search for biologically relevant interstellar molecules and the study of their formation processes; detection methods for potential spectroscopic evidence of biological activity; and finally the co-ordination of efforts in all these areas at the international level. Prof. Michael D. Papagiannis was elected President of the new commission and all IAU members who would like to join the new commission are requested to write to him at the Dept of Astronomy, Boston University, Boston, Massachusetts 02215, USA.

Following the First International Symposium on Radiation Physics, ISRP-1 (Calcutta 1974), interest has been expressed in forming an International Radiation Physics Society (IRPS) which could serve as the primary sponsor for further symposia in this interdisciplinary subject area. A pro-tem committee has now been elected to explore and to take the necessary actions to form such a society. The primary objective of which society would be the global exchange and integration of scientific information pertaining to radiation physics, with emphasis on (1) research, theoretical and experimental, in the field of radiation physics, (2) investigations of the physical aspects of the interactions of radiation with living systems, (3) education in radiation physics and (4) utilization of radiation for peaceful purposes. Further information from Prof. D.F. Jackson, University of Surrey, Guildford, UK.

A new scientific society, the American Society for Virology, has been officially inaugurated with the first of what will be annual meetings. W.K. Joklik of Duke University, was elected president of the society. The major purpose of the society will be the organization of annual meetings; the 1983 meeting being scheduled for Michigan State University next summer.

The International Society of Cryptozoology aims to be a focal point for the investigation, analysis, publication, and discussion of all matters related to animals of unexpected form or size, or unexpected occurrence in time or space. The society will promote the scientific examination of all evidence related to these matters. Although the society is primarily intended for biological scientists, membership is open to all interested persons. The society will publish both a quarterly newsletter and a scholarly, refereed journal, entitled *Cryptozoology*, which will appear once a year. Further information can be obtained from J. Richard Greenwell, International Society of Cryptozoology, PO Box 43070, Tucson, Arizona 85733, USA.

The Agricultural Research Council is to establish an Animal Breeding Liaison Group within the Edinburgh School of Agriculture, under the leadership of Prof. J.W.B. King. Its role will be to identify problems of current concern within the animal breeding industry and to promote the rapid uptake of research into farming practice. The group will liaise closely with other research establishments and relevant industrial organizations. Further information from M.L.S. Delaney, Agricultural Research Council, 160 Great Portland St, London W1, UK.